Enzyme Sources for Urinary Oxalate Measurements

Rukhsana Rahim

University of HUDDERSFIELD

Department of Chemical and Biological Sciences

Queensgate, Huddersfield, HD1 3DH

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An alternative source of oxalate oxidase (Oxox) was to be purified for use in a biosensor system to detect oxalate in patients suffering from primary hyperoxaluria (PH). Oxox has been isolated from a number of sources, however there is significant variation in the information available. Prior to the purification of Oxox, the properties of commercially available Sigma Aldrich Ltd. Oxox were researched.

An optimum enzymatic assay, substrate specificity, optimum buffer and optimum pH for Sigma Oxox were investigated prior to its purification. The purification of the enzyme led to the finding that Sigma Oxox was unstable in a number of salt buffers, and insoluble. To determine the ability of this enzyme to detect oxalate, amperometric analysis was undertaken, which demonstrated the Sigma Oxox based sensors' reusability and reproducibility.

Oxox from barley roots was successfully purified using a five step purification protocol, the findings obtained were in accordance with those published. The barley root extracts, after each purification procedure, were analysed amperometrically and highlighted the requirement for purification to optimise the analytical signal.

Over 140 sources were screened from a number of plant families, to detect the presence of Oxox activity. Three possible sources of Oxox were identified by level of Oxox activity and chosen for further purification: cabbage, carrot and mint leaves. The presence of Oxox in different varieties of these sources varied, illustrating the dissimilarities in Oxox, confirming the diversity of the data available on Oxox.

Oxox from cabbage, carrot and mint leaves were purified using the standardised protocol employed during the purification of Sigma and barley root Oxox. The studies undertaken led to the finding that the components of interest possessed dissimilar properties to documented Oxox. Cabbage, carrot and mint leaves oxalate oxidative components were found to be smaller than 700 kDa, extremely thermally stable, did not possess strong positively or negatively charged groups.

The identity of the oxalate oxidising components being Oxox was disproved. The possibility of the oxidising components being cofactors, or novel oxalate oxidising components were addressed, however, no distinct conclusion could be made.

ABTS	2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid)
AGT	Aminotranferase Glyoxylate
ASF	Ammonium Sulphate Fractionation
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CE	Capillary Electrophoresis
CM	Carboxymethyl
Da	Daltons
DEAE	Diethylaminoethyl
DEDTC	Diethyldithiocarbonate
DMA	N, N-diethylamiline
DTT	Dithiothreitol
E.C.	Enzyme Nomenclature
EDTA	Ethylenediaminetetraacetic Acid
EPR	Electron Paramagnetic Resonance
ESI MS	Electrospray Ionization Mass Spectrometry
ESWL	Extracorporeal Shockwave
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
FPLC	Fast Protein Liquid Chromatography
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectroscopy
Gox	Glucose Oxidase
GR	Glycoxylate Reductase
HPIC	High Performance Ion Chromatography
HPLC	High Performance Liquid Chromatography
HPLC-ER	High Performance Liquid Chromatography - Enzyme Reactor
HRP	Horseradish Peroxidase
kDa	Kilo Daltons
MBTH	3-methyl-2-benzothiazolinone hydrazone
MW	Molecular Weight
ND	None Detected

NHS	National Health Service
NMR	Nuclear Magnetic Resonance
Oxox	Oxalate Oxidase
PAGE	Polyacrylamide Gel Electrophoresis
PH	Primary Hyperoxaluria
PPO	Polyphenol Oxidase
PQQ	Pyrroloquinoline quinone
PTFE	Polytetrafluoroethylene
SDS	Sodium Dodecyl Sulphate
SF	Subcellular Fractionation
TFA	Trifluoracetic acid
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

The detection of oxalate in urine and blood is important in the diagnosis and management of certain clinical disorders. The pathological significance of oxalate in the formation of urinary stones has been known since the early 18th century [1]. The excessive consumption of oxalate rich foods and/or mutations in certain enzymes leads to elevated levels of oxalate in blood, plasma and urine. The accumulation of oxalate causes clinical disorders such as primary hyperoxaluria (PH), clinical renal failure and calcium oxalate hephrolithiasis. The objective of this introduction is to describe the disorder PH, the methods of detecting this disorder and to outline the aims of this research.

1.1 PRIMARY HYPEROXALURIA

The process of stone formation, urolithiasis, is also called nephrolithiasis. 'Nephrolithiasis' is derived from the Greek meaning 'nephros' (kidney) and 'lithos' (stone) [2]. Urolithiasis, known as kidney stones is a condition in which oxalate crystallises to form stones in the kidney, bladder and urethra [3-7]. The development of stones is related to decreased urine volume or increased excretion of stone forming components such as oxalate. Calcium oxalate kidney stones, known as hyperoxaluria, accounts for approximately 75 % to 85 % of the reported cases of urinary stones in patients [8, 9].

There are three main forms of hyperoxaluria: primary hyperoxaluria (types I and II), enteric hyperoxaluria, and idiopathic/mild hyperoxaluria [8]. Types I and II PH are caused by the deficiency in one of two liver enzymes, leading to the build up of oxalate. Type I PH sufferers are deficient in the peroxisomal enzyme alanine-glyoxylate aminotransferase (AGT), whereas type II patients are identified by a deficiency in the cytosolic enzyme glyoxylate reductase (GR) [10].

The metabolism of certain sugars and amino acids produces glyoxylate which is converted to two intermediate precursors: glycine or glycolate through AGT or GR liver enzymes respectively. Mutations/polymorphisms in the AGT or GR cause a shortage in these enzymes, preventing the conversion of glyoxylate to glycine or glycolate [10]. As a result, levels of glyoxylate increase, leading to its conversion to oxalate which in the presence of calcium leads to the precipitation of insoluble calcium oxalate forming kidney stones [11]. The schematic of the role of AGT and GR in types I and II PH during the metabolism of glyoxylate is shown in figure 1.1.

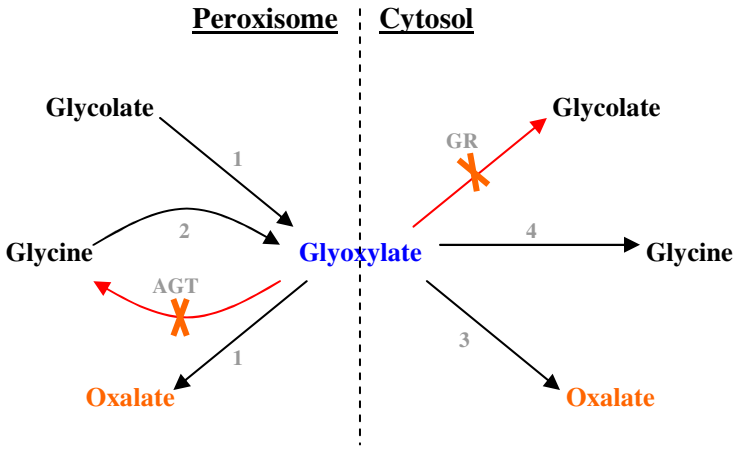


Figure 1.1: Schematic of the Accumulation of Oxalate.

1: glycolate oxidase, AGT: alanine-glyoxylate aminotransferase, 2: glycine oxidase, 3: lactate dehydrogenase, 4: glutamate-glyoxylate animotransferase, GR: glyoxylate reductase and X indicates malfunction [12].

Both AGT and GR enzymes are imperative since they determine the level of endogenous oxalate accumulated. The oxidative deamination of glycine to glyoxylate is catalysed by glycine oxidase, whereas the oxidation of glycolate to glyoxylate is catalysed by glycolate oxidase. Both reactions yield hydrogen peroxide as a byproduct [12].

1.1.1 Cause of Primary Hyperoxaluria

A number of polymorphisms have been identified in the AGT enzyme encoded by the AGXT gene. Of these polymorphisms, five point nucleotide substitutions have been identified, four of which are in the encoding region. Of these, two are synonymous, whereas the other two are nonsynonymous. Two intronic polymorphisms have also been identified, one of which is a polymorphic 74 bp duplication. Apart from the polymorphism that replaces proline with leucine 154C>T (P11L), none of the other reported polymorphisms have been known to have any functional significance.

Approximately 18 mutations have been identified in the AGXT gene. Most mutations are associated with particular enzymatic phenotypes, such as the presence or absence of AGT catalytic activity or immunoreactivity, peroxisome-to-mitochondrion mistargeting.

Polymorphism and mutations in type II PH have also been identified, however, this disorder has not been researched as well as type I PH. The GR mutations and polymorphisms either introduce signals disrupting the production of GR or alternatively, alter its structure resulting in the reduction or absence of GR activity [10].

1.1.2 Prevalence of Primary Hyperoxaluria

PH is uncommon, however, the incidence of this disorder has been difficult to determine. The reported incidence of PH varies, with some suggesting 1 % [13], and other between 2 % and 2.7 % [14]. Latta and Brodehl [14] estimated that 1 in 5 to 15 million children between 0 to 15 years old suffer from PH. Cochat *et al*. [15] calculated the prevalence of type I PH to be 2 per 1 million with the incidence rate of 1 per 100 thousand live births.

Since the incidence of type II PH is lower, these sufferers have been misdiagnosed with type I PH. Considering the late presentation of some patients suffering from type I and II PH, the true incidence is highly likely to be greater than the estimations outlined.

1.1.3 Diagnosis of Primary Hyperoxaluria

PH is diagnosed by pain at the site where the stone is causing irritation i.e. back and sides of the abdomen, lower front of the abdomen and groin area [2, 16]. The pain with PH is usually of sudden onset, very severe and intermittent and is known to be associated with nausea, vomiting, anorexia and depression.

Patients suffering from PH are diagnosed by detecting the concentration of oxalate in urine and other bodily fluids by a number of tests ranging from relatively simple enzymatic assays to complex chromatographic analysis. The urinary oxalate excretion rate in affected patients is typically 3 to 6 times higher than the normal level of approximately 450 μM [7, 8]. Oxalate can be measured directly using analytical techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE), or alternatively, quantified employing the enzyme oxalate oxidase (Oxox) for amperometric and enzymatic analysis, reviewed in chapters 1.2.1.1 and 1.2.2 later.

1.1.4 Treatment of Primary Hyperoxaluria

There are several factors which influence the ability to pass a stone, including, prior stone passage, prostate enlargement, pregnancy, and the size of the stone [2]. The majority of stones pass with no residual damage to the urinary system. If a stone does not pass, medication is prescribed aiding the solubility of calcium oxalate leading to its excretion with urine. The treatment of PH during its early stages is relatively simple with sufferers being advised to consume a low oxalate diet. A number of methods to remove stones from the kidney are routinely used in the medical profession. In the past, surgery was the only method of removing kidney stones [17], however, recently, research has led to new and improved methods such as:

- **Extracorporeal shockwave lithotripsy (ESWL)**: Shock waves break large stones into small stones, leading to their removal with urine [18, 19]
- **Percutaneous nephrolithotomy (Keyhole Surgery):** A small incision is made into the back forming a narrow tunnel through the skin, accessing the stone inside the kidney, leading to its removal [16, 20]
- **Ureteroscopy:** The uretheroscope is inserted into the urethra, up through the bladder and into the ureter where the stone is located. The ureteroscope aids the visualisation of the stone, followed by its removal [21, 22]

The recurrence rate of kidney stone formation is very high, therefore, prevention is very important especially in those with a high likelihood of redeveloping this condition. Drinking approximately 2 L of water per day [23] aids the excretion of kidney stones allowing most small stones to pass out with urine.

The treatment of PH varies according to the severity of the disorder and is dependent upon the circumstances of the patient as to which method of treatment is employed. The treatment of PH is financially straining on the National Health Service (NHS), therefore, rapid and effective methods of detecting the disorder are imperative to prevent further deterioration of health. The diagnosis of PH is not complex, time consuming or financially straining unlike its treatment, hence, it is vital to identify and diagnose the disorder effectively.

1.2 METHODS FOR DETECTING OXALATE

A number of methods are available to detect oxalate, the most common methods being amperometric, chromatography and spectrophotometric. Chromatography involves the detection of oxalate using HPLC, GC and CE. A summary of the methods available are listed in table 1.1.

Method	Reference
Amperometric	
Bound Oxox to acrylamine glass beads	**24- 28**
Chemiluminescence flow biosensor	**29**
Immobilised Oxox on silicone elastomer	**30**
Immobilised Oxox on graphite electrode	**31, 32**
Bi-enzyme biosensor	**33-36**
Chromatographic	
HPLC	**37-44**
HPLC-ER	**45, 46**
Reversed phase HPLC with chemiluminescence detection	**47-49**
HPIC	**41, 50-52**
Ion exclusion chromatography	**53**
GC	**41, 54-56**
GC-MS	**41, 54, 57, 58**
CE	**42, 52, 59-66**
Capillary ion electrophoresis	**64**
Isotachophoresis	**41**
Spectrophotometric	**31, 40-43, 49, 62, 63, 67-106**
Other	
Transparent Sol-Gel Monoliths	**81**
[^{14}C] oxalate decomposition	**107-109**
Electrochemiluminescence	**110**

Table 1.1: Methods for Detecting Oxalate.

Oxox: oxalate oxidase, HPLC: high performance liquid chromatography. ER: enzyme reactor, HPIC: high performance ion chromatography, GC: gas chromatography, MS: mass spectrometry, CE: Capillary Electrophoresis.

The methods listed in table 1.1 are routinely used to diagnose PH. In clinical diagnosis, rapidity, reliability, reproducibility and cost are all important factors, as a result, certain methods are preferred.

Amperometric and spectrophotometric detection of oxalate are very rapid with the analysis time ranging from 0.5 minutes to 5 minutes. On the other hand, HPLC analysis of oxalate varies from 3.8 minutes [62] to 7 minutes [65], GC analysis from 4 minutes [55] to 13.2 minutes [56] and CE analysis varying from 5.5 minutes [47] to 12 minutes [38]. Amperometric and spectrophotometric analysis are easy to perform, time efficient and do not require specialised training unlike HPLC, GC and CE analysis. Considering this, amperometric and spectrophotometric methods of detecting oxalate are preferred and are discussed further since they were the focus of attention during this research.

1.2.1 Amperometric Analysis

Biosensors are classified by the method used to achieve signal transduction. A biosensor has been defined as a *"compact analytical devise incorporating a biological or biologically-derived sensing element integrated within or intimately associated with a physicochemical transducer. The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes"* [111].

Biosensors are employed in a wide range of industries including agriculture, biotechnology, food, hygiene, medicine and pharmaceutical. Amperometric biosensors were first reported in 1962 with the development of enzyme electrodes by Leland Clark who wanted to make electrochemical sensors more intelligent by adding enzyme transducers as membrane enclosed sandwiches. Clark's electrode led to the development of the first glucose analyser in 1974 based on the amperometric detection of hydrogen peroxide at the platinum electrode as a result of the reduction of glucose oxidase (Gox) [111]. Amperometric biosensors have become more advanced since the first sensor with the development of reliable, reproducible, repeatable and mature biosensors for applications in many fields.

Amperometric transduction is a very sensitive method for detecting electrochemical species in biological samples. This technique employs three electrodes comprising a platinum

counter electrode, a working electrode and a reference electrode. Enzymes employed in this technique catalyse specific reactions leading to the production of electrochemically active species, which are in turn detected amperometrically. A constant potential is applied between the working and counter electrodes generating a current as a result of the oxidation or reduction of the target species.

The use of amperometric biosensors in the medical profession is of great interest since they have the ability to enhance the efficiency of detection and prognosis of many disorders. The most common sensor is the diabetes test kit which detects the level of blood glucose in patients suffering from diabetes [112]. An oxalate sensor has also been developed detecting levels of oxalate in patients suffering from PH, however, there is scope for improvement and its availability is limited, unlike the glucose sensor [113].

1.2.1.1 Amperometric Method for Detecting Oxalate

The amperometric biosensor system will be employed during this research to detect oxalate via Oxox. Oxox will be chemically immobilised/entrapped within a membrane in direct contact on one side with the sample solution, and on the other side with a suitable electrode: platinum for the detection of hydrogen peroxide.

The amperometric reaction involves an oxidation process, catalysed by Oxox generating hydrogen peroxide. The hydrogen peroxide is detected at the surface of the platinum electrode and is directly proportional to the concentration of oxalate in the sample solution analysed. Figure 1.2 illustrates the reaction taking place at the platinum surface of the electrochemical transduction device.

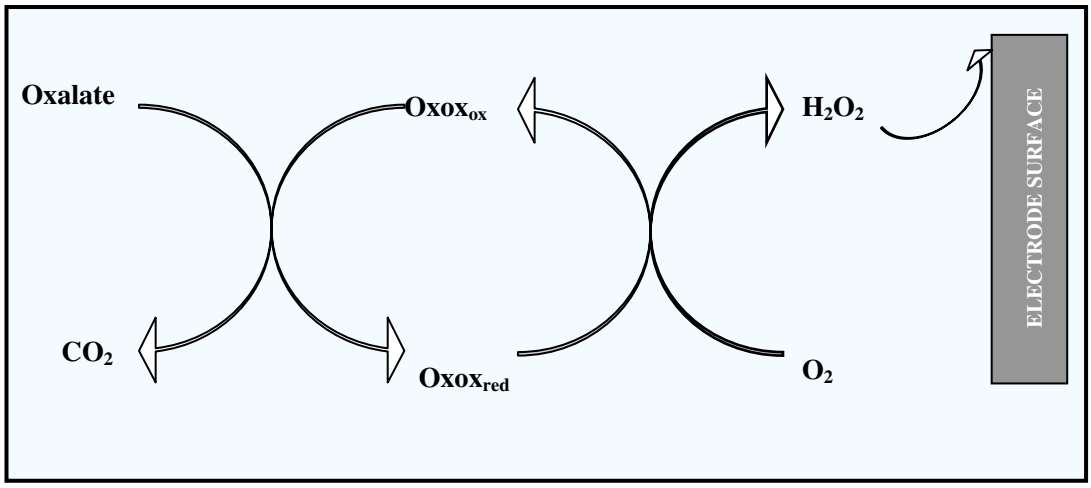


Figure 1.2: The Biochemical Reaction Occurring at the Electrode Surface.

Oxalate is oxidised by Oxox generating hydrogen peroxide, which in turn is detected at the platinum electrode surface. ox: oxidised and red: reduced.

The detection at the electrode surface can be via the oxidation of hydrogen peroxide or the reduction of oxygen. The oxidation reaction depicted in figure 1.2 takes place in the biological layer on the sensor.

The immobilisation of Oxox onto an electrode for oxalate measurements has been successfully achieved by a number of research groups [31-35]. The ability to detect oxalate, the rapid analysis time in addition to its specificity makes the amperometric biosensor system an interesting and appealing technique.

1.2.2 Spectrophotometric Assays for Detecting Oxalate

A number of established enzymatic assays have been optimised and investigated in detail by many research groups detecting oxalate utilising Oxox. The general principle of an oxalate assay involves the oxidation of oxalate by Oxox, generating hydrogen peroxide. The resultant hydrogen peroxide reacts with dye precursor(s) and horseradish peroxidase (HRP) yielding a coloured dye during the coupled assay:

$$\text{Oxalate} + O_2 \xrightarrow{\text{Oxalate Oxidase}} H_2O_2 + 2CO_2$$

$$H_2O_2 + \text{Dye Precursor(s)} \xrightarrow{\text{Horseradish Peroxidase}} \text{Coloured Dye} + H_2O$$

The four most common dye precursor based enzymatic assays used to detect oxalate are: ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid)), aminophenazone (4-Aminophenazone), σ-diansidine and MBTH (3-methyl-2-benzothiazolinone hydrazone) assays. Each oxalate coupled assay employs different dye precursor(s) and are monitored at ranging λ_{max}, shown in table 1.2.

Enzymatic Assay	Dye Precursor(s)	λ_{max} (nm)	Reference
ABTS	ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid)	650	**30, 73, 77, 85, 114**
Aminophenazone	4-Aminophenazone and Phenol	520	**24-27, 70, 72, 74, 79, 81, 104**
σ-Diansidine	σ-Diansidine	500	**115**
MBTH	MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMA (N,N-dimethylaniline)	578	**27, 28, 40, 69, 71, 78, 81, 86, 103, 106**

Table 1.2: A Selection of Enzymatic Assays for the Detection of Oxalate.

The dye precursor(s) and λ_{max} are shown alongside each oxalate coupled enzymatic assay.

The coloured dye generated during an enzymatic assay is directly proportional to the concentration of Oxox and oxalate in the assay mixture. Traditionally these enzymatic assays have been employed to measure the activity of Oxox and as a general identification protocol for oxalate.

Until 2003, Sigma Aldrich Ltd. were suppliers of an oxalate diagnostic kit based on a coupled oxidase colorimetric method utilising Oxox from barley roots. This kit employed two dye precursors: 3-methyl-2-benzothiazolinone hydrazone (MBTH) and N, N-diethylamiline (DMAB), however, this diagnostic kit was costly and inappropriate for analysing large numbers of urine samples due to the requirement for large amounts of Oxox. This method was uneconomical for routine measurements since commercially available Oxox is costly and the colorimetric method employed is also labour intensive. Therefore, there is a requirement for a cheaper source of Oxox and a reliable and rapid analytical method.

1.3 RESEARCH AIMS

The aim of this research was to design and fabricate a sensor for the determination of oxalate levels in urine to diagnose the onset of PH. Since the commercially available enzyme, Oxox, is costly, an alternative and novel source of Oxox was required. The isolated and purified enzyme was employed to detect oxalate enzymatically in addition to being immobilised using novel polymers for amperometric analysis.

A number of Oxox sources have been published with varying purification procedures and possess different characteristics and properties. The purification and characterisation of an alternative source of Oxox with significant activity will be undertaken to determine the following:

- Ionic stability
- Molecular mass
- Optimum pH
- Specific activity
- Substrate specificity
- Suitable enzymatic assay
- Thermal stability

Following the isolation and purification of a novel source of Oxox, a biosensor will be developed employing acrylate polymer emulsions to suitably immobilise/stabilise the enzyme onto a platinum electrode, in accordance with Mahmood [56].

The overall aim of this research is to develop a rapid and effective sensor to detect oxalate in patients suffering from PH. The oxalate sensor developed must be reusable, efficient, non-labour intensive, possess a long 'shelf life' and relatively simple to operate.

It is the objective of the following sections to review the literature available on Oxox which will be referred to throughout this research.

1.4 OXALATE OXIDASE

Oxalate oxidase (oxalate: oxygen oxidoreductase, E.C. 1.2.3.4) is a member of the Cupin superfamily of proteins [94] catalysing the oxidation of oxalate to form carbon dioxide and hydrogen peroxide. Oxox was first reported in filamentous fungi by Houget and Mayer in 1927 [116]. Since then, the presence of Oxox has been reported in numerous plants. The significance of Oxox in plants is not fully understood, with a number of hypotheses being introduced. It is thought the production of hydrogen peroxide during the oxidation of oxalate by Oxox destroys fungal toxins and microbes, serving as a defence mechanism [117-119], or it could be used in peroxidase catalysed cross linking reactions, strengthening cell walls [120].

Oxox has been purified and characterised from a variety of sources, ranging from fungi to plants. A summary of Oxox isolated from different sources is shown in table 1.3.

Sources and *Latin Name*	**Reference**
Amaranthus Leaves (*Amaranthus spinosus*)	**25, 74**
Banana Peel (*Musa paradisiacal* var. plantain)	**71, 105, 121**
Barley Leaves (*Hordeum vulgare*)	**67**
Barley Roots (*Hordeum vulgare*)	**44, 76, 78, 85, 86**
Barley Seedlings (*Hordeum vulgare*)	**84, 97, 122**
Beet Shoots, Stems and Leaves (*Beta vulgaris* L.)	**73, 76, 100, 103, 106, 123**
***Bougainvillea spectabilis* Leaves**	**99**
Ceriporiopsis subvermispora	**75**
Maize Roots (*Zea mays* L.)	**79**
Moss (*Minium affine)*	**124, 125**
***Pseudomonas* sp. OX-53**	**80**
Ryegrass (*Lolium perenne* L.)	**120**
Sorghum Leaves (*Sorghum vulgare* var. CSH-5, PC-6 and PC-1)	**24, 28, 70, 72, 82, 83, 104**
Sorghum Roots (*Sorghum vulgare* var. CSH-5)	**104, 126**
Tilletia controversa	**98**
Thale Cress *(Arabidopsis thaliana* L.)	**127**
Wheat Grains (*Triticum*)	**109**

Table 1.3: Sources of Reported Oxalate Oxidase.

The following section reviews the published data on the purification and characterisation of Oxox.

1.5 PURIFICATION OF OXALATE OXIDASE

Initially Oxox was only 'isolated' and characterised since the occurrence of this enzyme was of great interest. Most research groups isolated Oxox by employing only one isolation technique: subcellular fractionation [11, 29, 71, 79, 98, 99, 104, 123], whereas others isolated the enzyme by more complex four step protocols [23, 27]. An overview of the purification protocols performed to isolate and purify Oxox is highlighted in table 1.4.

	Method	Reference
ISOLATION	SF	**29, 71, 79, 98, 99, 104, 116, 123**
	SF, ASF	**26, 83, 126**
	SF, dialysis	**69**
	SF, affinity chromatography	**124**
	SF, thermal treatment, ASF	**67, 124**
	SF, anion exchange chromatography, affinity chromatography	**106**
	SF, anion exchange chromatography, cation exchange chromatography	**103**
	SF, ASF, cation exchange chromatography, gel filtration chromatography	**24**
	SF, ASF, anion exchange chromatography, gel filtration chromatography	**28**
PURIFICATION	SF, ASF, gel filtration chromatography	**73**
	SF, thermal treatment, ASF, isoelectric focussing	**84**
	SF, gel filtration chromatography, affinity chromatography	**44**
	SF, cation exchange chromatography, gel filtration chromatography	**75**
	SF, thermal treatment, ASF, anion exchange chromatography	**97, 122**
	SF, thermal treatment, affinity chromatography x 2	**78, 86**
	SF, ASF, cation exchange chromatography, gel filtration chromatography	**82**
	SF, ASF, anion exchange chromatography and gel filtration chromatography	**70, 72, 74**
	SF, thermal treatment, affinity chromatography, cation exchange chromatography, gel filtration chromatography	**85**
	SF, anion chromatography x 2, ASF, gel filtration chromatography	**80**

Table 1.4: Procedures Employed to Isolate and Purify Oxalate Oxidase.

Later, from the 1980s, efforts were concentrated on the purification of Oxox from a number of sources, however, the actual 'purification' of Oxox from these sources is questionable and has led to a plethora of purification protocols. The protocols available vary greatly, for example, Azarashvili *et al*. purified Oxox from beet shoots employing a three step purification protocol [73], whereas, Requena and Bornemann [85], and Koyama [80] purified Oxox from barley roots and *Pseudomonas* sp. OX-53 respectively using six step protocols.

The principle procedures employed to purify Oxox are subcellular fractionation (SF), thermal treatment, ammonium sulphate fractionation (ASF), affinity chromatography, ion exchange chromatography (anion and cation), gel filtration chromatography, chromatofocussing and isoelectric focussing. The general principles and the data available from the published literature of a selection of the purification procedures highlighted in table 1.4 will be discussed briefly in the following sections.

1.5.1 Subcellular Fractionation

The isolation of Oxox is essential to purify the desired enzyme, this is generally achieved by homogenisation which disrupts the cell walls by mechanical shearing, leading to the release of components into the surrounding environment. The released components after homogenisation generally fall into two categories, soluble and insoluble components.

A number of homogenisation tools have been used by research groups to homogenise sources of Oxox. A chilled pestle and mortar [24, 26-28, 70, 72, 78, 79, 83, 85, 104, 120] is the most favoured method to homogenise. However, a blender [33, 97, 99, 100, 103], a polyton homogeniser [84] and a polytetrafluoroethylene (PTFE) Potter-Elvehjem homogeniser [69] have also been used as homogenisation tools, though, not as often. The PTFE Potter-Elvehjem and polyton homogeniser are the best homogenisation tools since they possess rapid, efficient and highly physical shearing properties disrupting the homogenate by releasing additional components, however, they are not the conventional homogenisation tools. The blender on the other hand, does not possess high shearing capabilities, nevertheless, it is rapid and efficient.

To separate soluble components from the insoluble components within the homogenate, subcellular fractionation (SF) is necessary. SF separates components during centrifugation according to their molecular mass, shape and density [128]. After SF, the resultant pellet is a mixture of denser insoluble membrane bound components and the supernatant contains smaller sized soluble components. The soluble components remain untreated and are ready to analyse, however, the insoluble components require further treatment. The method of releasing insoluble components differs, with research groups employing acetone [73, 103, 121], digitonin [98], pepsin [79] or sodium deoxycholate [26, 74] treatment.

Table 1.5 indicates the centrifugal speeds employed to isolate Oxox from soluble (table 1.5a) and insoluble components (table 1.5b).

(a): Oxalate Oxidase Present within Soluble Components

Centrifugal Speed and Duration	Reference
1,000 x g to 4,000 x g for 20 to 30 minutes	**75, 84**
10,000 x g to 20,000 x g for 10 to 60 minutes	**44, 67, 78, 85, 87, 90, 95, 109, 124**
15,000 x g for 30 minutes	**24, 28, 70, 72, 82, 83, 104, 124, 126,**
59,000 x g for 60 minutes	**69, 97, 122**

(b): Oxalate Oxidase Present within Insoluble Components

Centrifugal Speed and Duration	Reference
1,000 x g for 10 minutes, pepsin treatment	**79**
15,000 x g for 15 to 20 minutes, sodium deoxycholate	**26, 74**
17,000 x g for 30 minutes, digitonin	**98**
14,000 to 30,000 x g for 30 to 40 minutes, acetone precipitation	**73, 103, 121**

Table 1.5: Centrifugal Speeds Employed to Isolate Oxalate Oxidase.

Oxalate oxidase isolated from the soluble (a) and insoluble membrane bound components (b).

Oxox has been reported to be located within both soluble and insoluble components of the cell, as a result, dissimilar SF parameters are expected. The centrifugal speed required to separate soluble components varies from 1,000 x g [84] to 59,000 x g [69, 97, 122] with 15,000 x g being the most favoured speed employed by many research groups. Similar

differences in centrifugal speeds have also been used to isolate insoluble components, ranging from 1,000 x g [79] to 30,000 x g [73].

1.5.2 Ion Exchange Chromatography

Ion exchange chromatography purifies components on the basis of charge, with the component of interest possessing a positive or negative charge, which bind to the opposing charge on either cation or anion exchange media [129-131]. Oxox has been purified using both ion exchange procedures, hence both are discussed below.

1.5.2.1 Cation Exchange Chromatography

Carboxymethyl (CM) cellulose is a commonly used cation exchanger and is composed of a cellulose bead support material with negatively charged carboxylic acid groups.

Charged components in the liquid phase pass through the column until they interact with a binding site in the cation media where positively charged molecules bind to the negatively charged CM cellulose beads [129-131]. Bound components elute from the column using a salt gradient, whereby the interactions between the media and bound species are disrupted. A range of cation exchange media have been used to purify Oxox and are outlined in table 1.6.

Cation Exchange Media	Reference
CM-Sepharose	**85**
Chelex 100 and AG-50	**69**
CM-52 cellulose	**77**
Mono S	**114**
Phosphocellulose	**75**

Table 1.6: Cation Exchange Media Employed to Purify Oxalate Oxidase.

1.5.2.2 Anion Exchange Chromatography

Diethylaminoethyl (DEAE) cellulose is a commonly used anion exchanger, the matrix is composed of positively charged amino groups, leading to its overall positive charge of the anion matrix.

The same principle applies to anion exchange chromatography with regards to opposing charged species binding to the charged groups. Negatively charged species bind to the column and are eluted by appropriate salt solutions which disrupt the binding interaction. A number of anion exchangers have been employed by research groups to purify Oxox from different sources, listed in table 1.7.

Anion Exchange media	Reference
DEAE Sephacel	**24, 27, 28, 70, 72, 74, 103, 106**
DE-52 Cellulose	**77, 80, 82**
DEAE- Sepharose CL-6B	**76, 80**
DEAE Cellulose	**97, 122**
Q Sepharose	**75**

Table 1.7: Anion Exchange Media Employed to Purify Oxalate Oxidase.

1.5.3 Affinity Chromatography

In affinity chromatography molecules bind to a ligand which is covalently attached to the affinity matrix. Sepharose is one of the most widely used support matrices possessing sites for covalent attachment of ligands. As molecules pass through the affinity column, certain components bind to the ligand, whereas others are eliminated as they pass through the column. Bound components have a high affinity for the ligand and are eluted when interactions between the ligand and the attached molecule are disrupted using appropriate solutions [129-131].

Many research groups employ affinity chromatography to purify Oxox, however, the media used varies with differing ligands attached, shown in table 1.8.

Affinity Media	Reference
Concanavalin A-Sepharose 4B	**78, 85, 86, 106, 121**
Sepharose CL 4B-Turquoise MX-G	**76**
Ultrogel A6R-Turquoise MX-G	**78, 86**

Table 1.8: Affinity Chromatography Media Employed to Purify Oxalate Oxidase.

1.5.4 Gel Filtration Chromatography

Gel filtration chromatography, also termed size exclusion chromatography, molecular exclusion chromatography, molecular sieve chromatography and gel permeation chromatography separates components based on hydrodynamic volume. The stationary phase of the gel filtration media is composed of semipermeable porous beads ranging in pore sizes. Smaller components have access to the mobile phase inside the beads and enter the pores slowing their movement through the column [129-131]. Large components can not fit inside the pores and only have access to the mobile phase between the beads, as a result, pass quickly through the column and elute first.

Different gel filtration media have been employed to purify Oxox from a number of sources and are listed in table 1.9.

Gel Filtration Media	Fractionation Range (kDa)	Reference
Bio-Gel A	10 to 500	**44, 80**
Protein PAK 3	10 to 300	**78**
Sephacryl S-200-HR	5 to 250	**77**
Sephadex G-100	4 to 150	**73**
Sephadex G-200	5 to 600	**24, 27, 28, 70, 72, 74, 75, 80, 82**
Superdex 200 HR	10 to 600	**85**

Table 1.9: Gel Filtration Media Employed to Purify Oxalate Oxidase.

The gel filtration media used by research groups to purify Oxox possess similar properties with regards to separating components according to their hydrodynamic volume, however, they differ in fractionation range. The most common gel filtration media used to purify Oxox is Sephadex G-200 since it possesses a wide fractionation range from 5 kDa to 600 kDa.

1.6 CHARACTERISATION OF OXALATE OXIDASE

Extensive research has been conducted with isolated and purified Oxox providing information regarding molecular mass, pH optima, thermal stability, substrate specificity, effect of flavins, salts and metal chelators. The properties and characteristics of purified

Oxox will be discussed to familiarise, investigate and enhance the knowledge of Oxox prior to its purification from alternative sources.

1.6.1 Molecular Mass and Subunit Composition of Oxalate Oxidase

Purified Oxox from different sources varies in molecular mass, with Pundir isolating Oxox from sorghum leaves with a molecular mass of 62 kDa [82] and Aguilar *et al*. with a molecular mass of 400 kDa [75]. Additionally, the number of subunits comprising Oxox differs from two [44, 70, 74, 84] to 8 [80, 85]. Table 1.10 is an overview of the reported molecular masses and subunit composition of purified Oxox from different sources.

	Molecular Mass (kDa)	**Subunits and Molecular Mass (kDa)**	**Reference**
Amaranthus Leaves	130	2 subunits of 65	**74**
Barley Roots	200	8 subunits of 25	**85**
Barley Roots	125	5 subunits of 26	**86**
Barley Seedlings and Roots	150	2 subunits of 75	**84, 44**
Ceriporiopsis subvermispora	400	6 subunits of 65.5	**75**
***Pseudomonas* sp. OX-53**	320	8 subunits of 38	**80**
Sorghum Leaves	120	2 subunits of 62	**72**

Table 1.10: The Molecular Masses of Purified Oxalate Oxidase.

The molecular mass of Oxox subunits varies from 25 kDa [85] to 75 kDa [44, 84] for 8 and two subunits respectively. Dissimilarities in molecular mass not only exist in Oxox purified from different sources, but also exists in the material from the same sources. For example, the molecular mass of Oxox purified from barley roots by Kostira and Clonis [86] is reported to be 125 kDa comprising five subunits, however, Oxox purified from the same source by Requena and Bornemann [85] is 200 kDa composed of 8 subunits.

1.6.2 pH Optima of Purified Oxalate Oxidase

Extensive information regarding the optimum pH of purified Oxox from different sources is available and vital since enzymes are more stable and active at a particular pH. A reduction or alternatively, an increase in pH may lead to lower rates of reaction. Table 1.11 summarises the optimum pH of purified Oxox from different sources.

	pH Optima	Reference
Amaranthus Leaves	3.5	**74**
Banana Peel	5.2	**121**
Barley Roots	3.5	**97**
Barley Roots	4.0	**78**
Barley Seedlings	3.2	**84**
Barley Seedlings	3.5	**97, 122**
Beet Shoots and Stems	4.0	**76, 103**
Beet Stems	4.5	**106**
***Bougainvillea spectabilis* Leaves**	6.8	**97**
Ceriporiopsis subvermispora	3.5	**75**
Maize	3.2	**79**
Moss	4.0	**125**
***Pseudomonus* sp. OX-53**	4.8	**80**
Sorghum Leaves	4.3	**82**
Sorghum Leaves	5.0	**70, 83**
Sorghum Leaves	4.0	**104**
Sorghum Leaves	4.5	**104**
Sorghum Roots	5.0	**104, 126**
Tilletia controversa	2.6	**98**

Table 1.11: The Optimum pH of Purified Oxalate Oxidase.

The optimum pH of purified Oxox ranges from pH 2.6 to pH 6.8, with most being towards the acidic range. Oxox purified from fungi [98] possesses a more acidic pH of 2.6, in comparison to Oxox purified from plants where the pH optima ranges from pH 3.5 to pH 5.0, with *Bougainvillea spectabilis* leaves being an exception at pH 6.8 [99].

1.6.3 Thermal Properties of Purified Oxalate Oxidase

There has been disagreement regarding the thermal stability of Oxox purified from different sources. Oxox purified from barley seedlings [84, 97] and barley leaves [67] are reported to be stable at 80 °C for 3 minutes. Barley roots [78, 85] have also been reported to be thermally stable, however, at a lower temperature of 60 °C for 10 minutes.

Banana peel Oxox isolated by Inamdar *et al*. in 1986 [121] retained 70 % of its activity after being thermally treated at 60 °C. Chiriboga [97] also found barley seedling Oxox to be

thermally stable at 60 °C for 3 minutes, however, treating at 89 °C for 3 minutes resulted in the loss of 51 % of the initial Oxox activity.

Bougainvillea spectabilis leaves Oxox [99] has been reported to be thermally unstable at 60 °C for 15 minutes since approximately 60 % of the activity was reported to be lost. Further thermal treatment at 80 °C to 100 °C led to the complete loss of activity. Additionally, Varalakshmi and Richardson [106] purified Oxox from beet stems and reported a loss of 50 % of its activity at 60 °C and 70 % at 70 °C.

Oxox purified from moss by Datta and Meeuse [125], and Suzuki and Meeuse [124] were reported to be thermally stable at 100 °C for 15 minutes with only a slight loss in Oxox activity. In contrast, Oxox purified from sorghum leaves [82] and three genotypes of sorghum [104] are not thermally stable above temperatures of 45 °C.

1.6.4 Substrate Specificity of Purified Oxalate Oxidase

When analysing urine samples for the detection of oxalate in patients suffering from PH, it is vital to eliminate possible interfering species. Therefore, the substrate specificity of Oxox is important when obtaining a 'true' representation of the concentration of oxalate in urine.

The substrate specificity of purified Oxox from a number of sources has been studied by replacing the conventional substrate, oxalate, with other potential substrates. The substrate specificity of Oxox purified from amaranthus leaves [74], barley seedlings [84], beet stems [44], *Ceriporiopsis subvermispora* [75], *Pseudomonas* sp. OX-53 [80] and sorghum leaves [72] have been reported. Relative percentage Oxox activities remaining after performing assays with alternative substrates are shown in table 1.12.

	% Activity Relative to Oxalic Acid	Reference
Oxalic Acid	**100, 100, 100**	**74, 80, 42**
Ascorbic Acid	0.36, 0, 0	**74, 80, 84**
L-Aspartic Acid	0.36	**84**
Citric Acid	0.44, <0.01, 0, 0	**72, 74, 80, 84**
L-Glutamic Acid	0.66, 0, 0	**72, 74, 84**
Glutaric Acid	0.72	**84**
Glycine	0.66, 0	**74, 84**
Glycolic Acid	0.55, 0	**80, 84**
Glycoxylic Acid	0.54, 3.7, 0, 1	**75, 80, 84, 106**
α-Ketoglutaric Acid	0.66, 0, 0	**74, 80, 84**
Maleic Acid	0.73, 1.9, 0	**72, 80, 84**
Malic Acid	0.73, 2.1, 0, 0	**74, 75, 80, 84**
Malonic Acid	0.66, 0	**75, 84**
Oxalacetic Acid	0.97, 0	**80, 84**
Oxalsuccinic Acid	1.09	**84**
Oxamide	0.73	**84**
Succinic Acid	0, 0, 0, 0	**72, 74, 80, 84**

Table 1.12: Possible Substrates for Purified Oxalate Oxidase.

All substrates investigated by Suguira *et al*. [84] with barley seedlings Oxox were at 0.6 mM with the exception of oxalsuccinic acid at 0.06 mM, Koyama [80] with *Pseudomonas* sp. OX-53 Oxox at 100 mM, Satyapal and Pundir [72] with sorghum Oxox at 0.5 mM, and [74, 75, 106] not stated.

The concentration of the substrates investigated in table 1.12 exceeds that of normal urinary excretion concentrations [132-134] with the exception of citric acid [135]. Only the studies undertaken by Koyama with citric acid [80] were greater than the normal excretion concentration of citric acid in urine.

The substrate specificity of Oxox investigated by numerous research groups led to less than 1 % relative activity with the exception of 1.09 % by oxalsuccinic acid [84], 1.9 % by maleic acid [80] and 3.7 % by glycoxylic acid [80]. The findings obtained by Suguira *et al*. [84], Koyama [80], Aguilar *et al*. [75], Varalakshmi and Richardson [106] and Satyapal and Pundir [72] were in agreement with each other with no contradictory findings. Therefore, it can be concluded that Oxox purified from amaranthus leaves [74], barley seedlings [84], beet stems [44], *Ceriporiopsis subvermispora* [75], *Pseudomonus* sp. OX-53 [80] and

sorghum leaves [72] are very specific to oxalate. As a result, the analysis of oxalate in urine using Oxox from these sources will generate 'true' results since the interfering compounds in urine: ascorbic acid, glutamic acid and glycolic acid will not affect the assay. Citric acid is also present in urine, however, the concentration of this compound used during specificity studies by Suguira *et al*. [84] and Satyapal and Pundir [72] were not sufficient. Therefore, it is not conclusive whether citric acid will affect the assay when analysing urine samples.

1.6.5 Effect of Flavins on Purified Oxalate Oxidase

The effect of flavins has been investigated by numerous research groups to further characterise purified Oxox. The most common flavins researched are flavin adenine dinucleotide (FAD) derived from vitamin B_{12}, flavin mononucleotide (FMN) synthesised from riboflavin and riboflavin (7,8-dimethyl-10(1'-D-ribityl)isoalloxazin) known as vitamin B_2. An overview of the published data regarding the effect of FAD, FMN and riboflavin on Oxox activity is shown in table 1.13.

Conflicting findings have been reported regarding the effect of flavins on purified Oxox. Studies with sorghum leaves in 1983 by Pundir and Nath [83] led to the finding that FAD had no effect on sorghum leaves Oxox activity. However, later in 1990, Pundir [82] reported FAD stimulated sorghum Oxox activity by 500 %. Pundir suggested this discrepancy was likely to be due to the removal of the cofactor during purification.

FAD, FMN and riboflavin did not significantly affect Oxox activity in amaranthus leaves [74], barley roots [78] and beet shoots [73]. However, flavins enhanced the activity in barley seedlings [97] and *Pseudomonas* sp. OX-53 [80].

	Flavin	Final Concentration (mM)	% Activity Stimulation (+) or Inhibition (-)	Reference
Amaranthus Leaves	FAD	1	0	**74**
	FMN	1	-11	**74**
	Riboflavin	1	-4	**74**
Barley Roots	FAD	1	0	**78**
	Riboflavin	1	0	**78**
Barley Seedlings	FAD	0.01	+32	**97**
	FMN	0.01	+45	**97**
	Riboflavin	0.01	+95	**97**
Beet Shoots	FAD	0.1	0	**73**
	FMN	0.1	0	**73**
	Riboflavin	0.1	0	**73**
Beet Stems	FAD	1	+143	**106**
	FMN	0.5	+591	**106**
	Riboflavin	1	+697	**106**
***Pseudomonus* sp. OX-53**	FAD	0.1	+66	**80**
	FMN	0.1	+88	**80**
	Riboflavin	0.1	+111	**80**
Sorghum Leaves	FAD	0.5	+31	**72**
	FMN	0.5	+57	**72**
	Riboflavin	0.5	-1	**72**
Sorghum Leaves	FAD	0.1	0	**83**
	FAD	0.1	+500	**82**
Sorghum Roots	FAD	0.5	0	**126**
	FMN	0.5	+25	**126**
	Riboflavin	0.5	+30	**126**
Tilletia contraversa	FAD	-	0	**98**
	FMN	-	+190	**98**
	Riboflavin	-	+260	**98**

Table 1.13: The Effect of FAD, FMN and Riboflavin on Purified Oxalate Oxidase.

Strong flavin stimulation was also observed in beet stems [106] by Varalakshmi and Richardon where Oxox activity was stimulated by 143 % (FAD), 591 % (FMN) and 697 % (riboflavin). Oxox purified from sorghum roots by Pundir and Kuchhal [126] led to the stimulation of Oxox activity by riboflavin by 30 % and 25 % by FMN, however, no effect was observed with FAD. Further studies performed by Satyapal and Pundir [72] with sorghum leaves Oxox generated similar results with the slight stimulation of Oxox activity by only two flavins investigated. As a result, Pundir and Kuchhal [126] concluded flavins did not affect Oxox activity. Surprisingly, not all flavins investigated by Vaisey *et al*. [98] stimulated Oxox activity in *Tilletia contraversa*.

1.6.6 Effect of Salts on Oxalate Oxidase Activity

The effect of various salts on Oxox purified from amaranthus leaves [74], barley roots [78], barley seedlings [84, 97], *Bougainvillea spectabilis* leaves [99], maize roots [5], moss [124], *Pseudomonas* sp. OX-53 [80], sorghum leaves [72, 82, 83, 104] and sorghum roots [9] have been investigated by many research groups. This section reviews the published data detailing the effect of salts on Oxox purified from the above sources.

1.6.6.1 Effect of Sodium Chloride

Oxox purified from amaranthus leaves [74], barley roots [78], barley seedlings [84], *Bougainvillea spectabilis* leaves [99], maize roots [79], moss [124], sorghum leaves [72, 82, 83, 104] and sorghum roots [9] have been investigated to determine the effect of sodium chloride on the purified enzymes. To study the effect of sodium chloride, Oxox was either washed with sodium chloride [79], or alternatively, differing concentrations of this salt were added to the assay mixture [70, 82, 83]. The results collated from the literature are summarised in table 1.14.

	Final Concentration (mM)	% Activity Stimulation (+) or Inhibition (-)	Reference
Amaranthus Leaves	1	0	**74**
Barley Roots	1	-14	**78**
Barley Seedlings	1	-57	**84**
***Bougainvillea spectabilis* Leaves**	100	-50	**99**
Maize Roots	1000	-10	**79**
Moss	0.2	-32	**124**
Sorghum Leaves	0.5	0	**82**
Sorghum Leaves	250	0	**83**
Sorghum Leaves	200	0	**104**
Sorghum Leaves	0.01	0	**72**
Sorghum Roots	0.5	+2	**126**

Table 1.14: The Effect of Sodium Chloride on Purified Oxalate Oxidase.

Considering the concentration of sodium chloride investigated ranges from 0.01 mM to 1 M, the resultant information cannot be directly compared, however, gives an indication

regarding the effect of this salt at differing concentrations on Oxox activity from varying sources.

The effect of sodium chloride on purified Oxox is dissimilar, with some being inhibited by this salt and others unaffected. Oxox purified from amaranthus leaves [74], maize roots [79], sorghum leaves [72, 82, 83, 104] and sorghum roots [126] were unaffected by sodium chloride with 100 % of the relative activity remaining with an exception of 90 % for maize roots [79] and 98 % for sorghum roots [126]. These result led Vuletić *et al*. [79] to conclude sodium chloride had no effect on Oxox purified from maize on the basis that it not stimulate Oxox activity. Conversely, Oxox activity was inhibited in barley roots by 14 % [78], barley seedlings by 57 % [84], *Bougainvillea spectabilis* leaves by 50 % [99] and moss by 32 % [124].

In 1990, Pundir [82] suggested sodium ions were responsible for the loss of Oxox activity in *Bougainvillea spectabilis* leaves and barley enzymes. Later publications with Nath [83], Pundir suggested Oxox purified from these sources were in fact sensitive to sodium ions.

1.6.6.2 Effect of Anions and Cations

The effect of many salts on Oxox purified from different sources has been investigated to obtain an enhanced understanding of the enzyme. The effect of halides such as F^-, Cl^-, Br^- and I^- as well as the effect of various anions (N_3^-, CO_3^{2-}, HCO_3^-, NO_3^-, SO_4^{2-}) and cations (Al^{2+}, Ag^+, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, Pb^{2+}, Sn^{2+}, Sr^{2+}, VO^{3-}, Zn^{2+}) on purified Oxox has been studied. The resultant information regarding the effect of anions and cations was collated and tabulated in tables 1.15 (anion) and 1.16 (cation).

		Final Concentration (mM)	% Activity Stimulation (+) or Inhibition (-)	Reference
Amaranthus Leaves	F^-	1	0	**74**
	Br^-	1	-6	**74**
	CO_3^{2-}	1	-8	**74**
	HCO_3^-	1	-17	**74**
	NO_3^-	1	-2	**74**
	SO_4^{2-}	1	0	**74**
Barley Seedlings	F^-	1	-60	**84**
	Br^-	1	+14	**84**
	I^-	1	-86	**84**
	N_3^-	1	-98	**84**
	CO_3^{2-}	1	-48	**84**
	NO_3^-	1	-84	**84**
	SO_4^{2-}	1	-25	**84**
Barley Roots	Cl^-	1	-14	**78**
	NO_3^-	1	-3	**78**
	SO_4^{2-}	1	0	**78**
Moss	F^-	2	-89	**124**
	Br^-	2	-40	**124**
	I^-	2	-80	**124**
***Pseudomonus* sp. OX-53**	Cl^-	1	+6	**80**
	Br^-	1	-57	**80**
	I^-	1	-94	**80**
Sorghum Roots	Cl^-	0.5	+200	**126**
	SO_4^{2-}	0.5	+15	**126**
Sorghum Leaves	Cl^-	0.5	0	**72**
	Br^-	0.5	-7	**72**
	I^-	0.5	-14	**72**
	N_3^-	0.5	-57	**72**
Tilletia Contraversa	F^-	0.16	-50	**98**

Table 1.15: The Effect of Anions on Purified Oxalate Oxidase.

Pundir and Satyapal [70], and Kotsira and Clonis [78] purified Oxox from sorghum leaves and barley roots respectively and found that NO_3^- did not affect the activity, unlike Oxox purified from barley seedlings [84] which was inhibited by 84 %. Goyal *et al*. [74] was in agreement with Pundir and Satyapal [70] regarding the effect of NO_3^- since he also suggested amaranthus leaves Oxox was NO_3^- insensitive.

Goyal *et al*. [74] also investigated the effect of various anions on amaranthus leaves Oxox and led to the finding that HCO_3^- inhibited Oxox activity by 17 %, however, other anions such as F^-, Br^-, CO_3^- and SO_4^{-2} had no effect.

The effect of Cl^- on Oxox purified from barley roots [78], *Pseudomonas* sp. OX-53 [80] and sorghum leaves [72] has also been researched and found that Cl^- did not affect Oxox activity. Pundir and Satyapal [70] and Goyal *et al.*'s [74] studies with sorghum and amaranthus leaves respectively demonstrated Oxox were not sensitive to Cl^- since the enzyme activities were unaffected. Contrary to this, Cl^- profoundly stimulated Oxox activity in sorghum roots [126] by 200 %.

The effect of halides on Oxox purified from amaranthus leaves [74], barley seedlings [84], moss [124], *Pseudomonas* sp. OX-53 [80], sorghum leaves [72] and *Tilletia contraversa* [98] has been researched and contrasting findings have been reported. Halides insignificantly affected the activity in sorghum leaves [72], however, Oxox activity was inhibited and stimulated in barley seedling [84], moss [124] and *Pseudomonas* sp. OX-53 [80].

		Final Concentration (mM)	% Activity Stimulation (+) or Inhibition (-)	Reference
Barley Seedlings	Ag^+	1	-22	**84**
	Ca^{2+}	1, 7.5	+14, -3	**84, 97**
	Cu^{2+}	1, 7.5	+36, -59	**84, 97**
	Hg^{2+}	1	-73	**84**
	Mn^{2+}	1, 7.5	-3, -44	**84, 97**
	Na^+	1	-11	**84**
	Ni^{2+}	1	+51	**84**
	Pb^{2+}	1	+12	**84**
	Zn^{2+}	1	+36	**84**
Barley Seedlings	Ba^{2+}	7.5	-32	**97**
	Fe^{2+}	7.5	-100	**97**
	Sr^{2+}	7.5	-2	**97**
Barley Roots	Ca^{2+}	1	+71	**78**
	Cu^{2+}	1	+10	**78**
	Fe^{2+}	1	-99	**78**
	Pb^{2+}	1	+157	**78**
***Bougainvillea* Leaves**	Ca^{2+}	1	-40	**99**
***Pseudomonus* sp. OX-53**	Cu^{2+}	0.1	-36	**80**
	Fe^{2+}	0.1	-6	**80**
	Hg^{2+}	0.1	-47	**80**
	Mg^{2+}	0.1	-3	**80**
	Na^+	0.1	-10	**80**
	Mn^{2+}	0.1	+75	**80**
	Zn^{2+}	0.1	-12	**80**
Sorghum Roots	Cu^{2+}	0.5	+210	**126**
Sorghum Leaves	Cu^{2+}	0.5	+200	**72**

Table 1.16: The Effect of Cations on Purified Oxalate Oxidase.

Amaranthus leaves [74], moss [124] and sorghum leaves [83] Oxox activity was insignificantly affected by K^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, VO^{3-} and Zn^{2+}. Conversely, Satyapal and Pundir [72], and Pundir and Kuchhal [126] demonstrated Oxox from sorghum leaves and roots were strongly stimulated by only Cu^{2+} by 200 % and 210 % respectively.

Barley seedling Oxox was found to be very sensitive to changes in ionic strength, the divalent affects of the cations could not be explained by Chiriboga [97]. This led to the suggestion that the inhibition was likely to be caused by a mixture of reactions between the metal, protein, substrate and the enzyme-substrate complex. It was proposed the divalents Cu^{2+} and Ca^{2+} had acted on the substrate as well as the enzyme causing the adverse effects [97].

The effect of bivalent metal ions on *Bougainvillea spectabilis* leaves Oxox activity investigated by Srivastava and Krishnan [99] demonstrated Ca^{2+} inhibited the activity by 40 %, whereas Mn^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+} and Ni^{2+} had no effect. Goyal *et al*. [74] performed similar studies with Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+} and Zn^{2+}, however, only Fe^{2+} stimulating Oxox activity purified from amaranthus leaves.

Vuletić *et al*.'s [79] studies with Al^{3+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+} on maize Oxox demonstrated these divalent metals did not affect the activity. Similar findings were obtained by Bornemann [114] during the investigation of the effect of Fe^{2+}, Fe^{3+} and Zn^{2+}on commercially available Oxox from Boehringer Mannheim.

The effect of manganese ions on Oxox activity is of great interest since studies performed by Bornemann [85] suggested Oxox from barley roots contains a manganese cofactor. Manganese ions have been reported not to affect Oxox activity in barley seedlings [84], maize [79] and Boehringer Mannheim Oxox [114]. In contrast, manganese stimulated Oxox activity in *Pseudomonas* sp. OX-53 by 75 %, however, Koyama concluded Mn^{2+} only *slightly* stimulated Oxox activity [80].

Bornemann [85] preincubated barley root Oxox with manganese chloride and observed no increase in Oxox activity. This led Bornemann to suggest either the enzyme had its full

complement of Mn^{2+}, or it had not reversibly bound the ion in its fully folded form. He concluded the latter was more likely since extensive dialysis performed in the absence of Mn^{2+} did not lead to the loss of Oxox activity [85].

1.6.6.3 Effect of Metal Chelators on Purified Oxalate Oxidase

The effect of metal chelators on purified Oxox has been studied extensively by many research groups to determine the metal ion requirement of Oxox.

A number of ion chelators have been investigated including, α, α'- dipyridyl: an Fe^{2+} and Cu^{2+} chelator, diethyldithiocarbamate (DEDTC): a Cu^{2+} chelator, ethylenediaminetetraacetic acid (EDTA): a non-specific metal ion chelator, and 8-hydroxyquinoline: a Mg^{2+} and Ca^{2+} chelator. The effect of these ion chelators on Oxox purified from amaranthus leaves [74], barley roots [78], barley seedlings [84, 122], *Bougainvillea spectabilis* leaves [99], moss [124], maize [79], *Pseudomonas* sp. OX-53 [80], sorghum leaves [72, 82, 83] and sorghum roots [126] is shown in table 1.17.

The effect of metal chelators on Oxox purified from various sources is contradictory, with the activity of Oxox in some cases being inhibited, retained or stimulated by metal chelators.

The inhibition of Oxox activity by α, α'- dipyridyl was more prominent in Oxox purified from sorghum leaves by 52 % [83], with the remaining sources of Oxox not being inhibited as strongly. For example, α, α'- dipyridyl inhibited Oxox purified from barley seedling by 21 % [122], moss by 13 % [124], *Pseudomonus* sp. OX-53 by 31 % [80], sorghum leaves by 30 % [82] and sorghum roots by 38 % [126].

EDTA had no effect on Oxox purified from amaranthus leaves [74], barley roots [78] and maize [79] since 100 % of the relative activity remained. On the other hand, EDTA inhibited Oxox activity in *Bougainvillea spectabilis* leaves by 44 % [99], *Pseudomonas* sp. OX-53 by 42 % [80], sorghum leaves by 30 % [83] and 54 % [72].

	Compound	Final Concentration (mM)	Relative Activity (%)	Reference
Amaranthus Leaves	DEDTC	1	-73	**74**
	EDTA	1	0	**74**
	8-Hydroxyquinoline	1	0	**74**
Barley Roots	EDTA	1	-8	**78**
Barley Seedlings	α, α'- Dipyridyl	0.2	-21	**122**
	EDTA	1, 0.2	+3, +31	**84, 122**
	8-Hydroxyquinoline	1, 0.2	+86, +333	**84, 122**
***Bougainvillea spectabilis* Leaves**	EDTA	6.7	-44	**99**
	8-Hydroxyquinoline	3.3	+100	**99**
Moss	α, α'- Dipyridyl	2	-13	**124**
	8-Hydroxyquinoline	2	0	**124**
Maize	EDTA	5	0	**79**
***Pseudomonus* sp. OX-53**	α, α'- Dipyridyl	0.1	-31	**80**
	EDTA	0.1	-42	**80**
	8-Hydroxyquinoline	0.1	-41	**80**
Sorghum Leaves	α, α'- Dipyridyl	0.1, 0.5	-52, -30	**82, 83**
	EDTA	0.1, 0.5	-30, -54	**72, 83**
	8-Hydroxyquinoline	0.1	-52	**83**
Sorghum Roots	α, α'- Dipyridyl	0.5	-38	**126**
	DEDTC	0.5	-30	**126**
	EDTA	0.5	-38	**126**
	8-Hydroxyquinoline	0.5	+50	**126**

Table 1.17: The Effect of Metal Chelators on Purified Oxalate Oxidase.

The effect of α, α'- dipyridyl, DEDTC, EDTA and 8-hydroxyquinoline on Oxox purified from different sources.

DEDTC inhibited Oxox activity in amaranthus leaves [74] by 73 % and sorghum roots by 30 % [126]. Amaranthus leaves [74] and moss [124] Oxox activity remained unaltered after the addition of 8-hydroxyquinoline, however, Oxox activity was inhibited in *Pseudomonus* sp. OX-53 by 41 % [80] and sorghum leaves by 52 % [83]. Oxox activity in barley seedlings was stimulated by 86 % [84] and 333 % [122], in *Bougainvillea spectabilis* leaves by 100 % [99] and sorghum roots by 50 % [126] by 8-hydroxyquinoline. Chiriboga [122] suggested the stimulation by 8-hydroxyquinoline may be due to the chelation of metallic impurities which may have been inhibiting the system, the formation of some kind of chelate that could transport electrons, or some action of the active centre of the enzyme.

Inhibition studies performed by Suzuki and Meeuse in 1965 [124] showed that thiocynate, azide and DEDTC were the most powerful inhibitors, however, the effect of other inhibitors such as α, α'- dipyridyl and 8-hydroxyquinoline (shown in table 1.17) were not as severe. These finding led them to suggest that a heavy metal constituent may be present in moss Oxox.

1.6.6.4 Is Oxalate Oxidase a Flavoprotein or a Metalloprotein?

The very first Oxox to have been reported to require a cofactor was isolated from barley seedling by Fodor *et al*. in 1930 which required Kocksaft yeast to catalyse the reaction [124]. Following this, Datta and Meeuse in 1955 [125] stated moss Oxox required riboflavin or FAD as a cofactor to catalyse the reaction. Chiriboga [97] agreed that some kind of flavin was probably associated with barley seedling Oxox to form hydrogen peroxide, however, he had no direct evidence for this. Vaisey's [98] findings were in accordance with those published by Datta and Meeuse [125] in that *Tilletia contraversa* Oxox activity was only stimulated by two flavins: riboflavin and FMN.

Later in 1964 Suzuki and Meeuse [124] disagreed with the information regarding the properties of purified Oxox since initial studies were only performed using crude preparations of the enzyme. This led Suzuki and Meeuse to suggest moss Oxox was a Fe^{3+} containing enzyme.

Flavins have been reported to stimulate Oxox purified from barley seedlings [97], beet stems [106], *Pseudomonas* sp. OX-53 [80], sorghum leaves [72, 82], sorghum roots [126] and *Tilletia contravera* leaves [98]. These findings led many research groups to classify Oxox as a flavoprotein on the basis of its stimulation by flavins.

Later, the involvement of metal ions was proposed, however, there was controversy regarding the identity of the metal ion associated with Oxox purified from different sources. Satyapal and Pundir [72], Suguira *et al*. [127], Pundir and Kuchhal [126], Pundir [72] and Lane [136] suggested Oxox was a copper requiring enzyme. On the other hand, Pundir [82], Suzuki and Meeuse [124], and Pundir and Nath [83] suggested the requirement of iron. To confuse matters further, some research groups have suggested the involvement of calcium and even lead [78, 114].

It was later in 1998 when Bornemann concluded that the suggestions made by Lane [136] and Pundir [72] regarding the involvement of a copper cofactor, or iron by Suzuki *et al*. [124] and Pundir [82] provided no direct evidence. Bornemann eliminated the suggestion of copper, calcium, iron and lead as candidate cofactors associated with Oxox by performing metal analysis using inductively coupled plasma technique and electron paramagnetic resonance (EPR) spectroscopy. These finding led Bornemann to conclude Oxox purified from barley roots contained only mononuclear Mn^{2+}, he further stipulated Mn^{2+} was likely to be responsible for catalysis, rather than just having a structural role [85].

Bornemann's findings regarding the presence of manganese were in accordance with Koyama's observations made earlier in 1988. Atomic absorption performed by Koyama on *Pseudomonas* sp. OX-53 Oxox led him to conclude Oxox was a manganese containing metalloprotein [80].

1.7 PURIFICATION OF OXALATE OXIDASE FROM AN ALTERNATIVE SOURCE

Oxox has been purified from three classes of organisms: fungi, bacteria and plants, with the majority of Oxox sources being plants and some being isolated from the same family. The sources of Oxox are identified in table 1.18 along with the families they originate from.

Some sources of Oxox are derived from the same family, for example, barley, maize, ryegrass, sorghum and wheat have been reported to contain Oxox and are all members of the *Poaceae* family. Two members of the *Amaranthaceae* family: amaranthus and beet have also been reported to contain Oxox. The presence of Oxox in multiple members of a family implies certain families may be more likely to possess Oxox than others.

Datta and Meeuse [125] appear to be the only research group to have investigated the presence of Oxox in a family from which one source has been reported to possess Oxox: moss. A dozen species were screened from the *Bryales* family since moss, also a member of this family, had been reported to possess Oxox activity. The species screened from the *Bryales* family possessed Oxox activity, however, the presence of the enzyme is some species was more prevalent than others [125].

Family	Source
Amaranthaceae	Amaranthus Leaves
	Beet Shoots
Brassicaceae	Thale Cress
Bryophyta	Moss
Fungi	*Ceriporiopsis subvermispora*
Musaceae	Banana Peel
Nyctaginaceae	*Bougainvillea spectabilis* Leaves
Poaceae	Barley Leaves
	Barley Roots
	Barley Seedlings
	Maize Roots
	Ryegrass
	Sorghum Leaves
	Sorghum Roots
	Wheat Grains
Proteobacteria	*Pseudomonas* sp. OX-53
Tilletiaceae	*Tilletia controversa*

Table 1.18: Purified Sources of Oxalate Oxidase.

Pundir [104] investigated the presence of Oxox in three genotypes of sorghum. These studies led to the finding that all three genotypes contained Oxox, however, the location of Oxox varied in the order of leaves>stems> roots in PC-6 and PC-1 sorghum varieties, but the order was reversed in CSH-5.

Since the majority of Oxox sources are plants, alternative sources of Oxox in plants will be investigated to achieve the aim of this research by screening a number of commercially available and cultivated plants. The *Apiaceae, Asteraceae*, *Brassicaceae, Cucurbitaceae, Fabaceae, Lamiaceae*, *Poaceae* and *Solanaceae* families will be screened to identify and isolate a novel source of Oxox. Finally, the purified enzyme will be used to develop a biosensor system for diagnostic use as described in chapter 1.3.

CHAPTER 2

METHODOLOGIES AND STANDARDISATION OF PROCEDURES

The methods employed during the purification and characterisation of Oxalate oxidase (Oxox) and the initial sensor work are discussed in this chapter. The general protocol for each method employed during this research is discussed in addition to preliminary studies undertaken to standardise and optimise experimental conditions.

2.1 MATERIALS

Glucose oxidase type II-S (E.C 1.1.3.4, from *Aspergillus niger*), oxalate oxidase (E.C 1.2.3.4, from barley roots), bovine serum albumin 98 %, α-D-mannopyroside 99 %, sodium sulphate and the reagents for enzymatic assays: 3-methyl-2-benzothiazolinone hydrazone (MBTH), N,N-dimethylaniline (DMA) 99 %, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS) 98 % diammonium salt, σ-diansidine and phenol were obtained from Sigma Aldrich Ltd.

Chromatoraphic media carboxymethyl cellulose, diethylaminoethyl cellulose, Concanavalin-A Sephacryl 4B, Sephacryl S-200-R, Sephadex G10, G15 and G25 were obtained from Sigma Aldrich Ltd. Gel filtration MW-GF-200 molecular weight markers were also obtained from Sigma Aldrich Ltd. Bio-Gel P2 media was obtained from Bio Rad Ltd. XK and C type chromatography columns were obtained from Amersham Pharmacia.

Sodium phosphate buffers were prepared using di-sodium orthophosphate (dodecahydrate), and sodium dihydrogen orthophosphate. Potassium phosphate buffers were prepared using potassium dihydrogen phosphate and dipotassium hydrogen phosphate, all of which were obtained from Sigma Aldrich Ltd. Acetate buffers were prepared with HPLC grade acetic acid and sodium acetate trihydrate. HPLC grade sodium hydrogen carbonate and sodium carbonate were obtained from Sigma Aldrich Ltd to make carbonate buffers.

Silver nitrate staining kit, Coomassie Blue and Bicinchoninic acid protein assay kit were purchased from Sigma Aldrich and NuPAGE® Bio-Tris polyacrylamide Gels were purchased from Invitrogen Ltd.

Manganese chloride (manganese (II) chloride tetrahydrate LR) was purchased from May and Baker Ltd (M&B) Dagenham, England. Potassium chloride 99.8 %, magnesium

chloride 98 %, 4-aminophenazone, succinic acid 99 %, AnalaR grade oxalic acid, sodium deoxycholate and AnalaR grade ammonium sulphate were obtained from BDH.

The *Poaceae* family crops 'Dandy' barley, rye, sorghum, 'summer' and 'winter' wheat were purchased from Monsanto UK Ltd. Gravel and silver sand were purchased from B&Q plc. and fertilised coir compost was obtained from Champak Ltd.

Dialysis tubing with a molecular cut off of 12 kDa was purchased from Medicell International Ltd.

2.2 INSTRUMENTATION

A Shimadzu UV-160A, UNICAM UV/Vis and Varian Cary 1E UV/Vis spectrophotometers were used throughout this project. A RC5C centrifuge with a SS34 rotor was used to centrifuge extracts homogenised with a Braun® hand blender, Potter-Elvehjem homogeniser and pestle and mortar.

Chromatographic procedures were performed using the ÄKTA prime® and ÄKTA FPLC instruments.

Electrochemical measurements were carried out on PGstat 10 potentiostat (Autolab, Eco Chemie) using the General Purpose Electrochemical System software (version 4.4).

Seedlings were cultivated in Sanyo Gallenkamp Plc. Fititron 600H and 6143, and Fision 140 G2 growing chambers.

2.3 SOURCES SCREENED FOR OXALATE OXIDASE

A number of sources from the *Asteraceae*, *Apiaceae, Brassicaceae, Cucurbitaceae, Fabaceae, Lamiaceae*, *Poaceae* and *Solanaceae* families were screened to isolate and identify the presence of Oxox. Most sources screened were either cultivated or purchased commercially from Asda Ltd., Tesco plc. and Sainsburys Ltd.. Where possible, the leaves, peels, roots, shoots and seedlings of some sources were investigated in an attempt to locate possible Oxox. The sources investigated from each family are listed in table 2.1.

Asteraceae Family

Common Name	Latin Name
Radicchio *	*Cichorium intybus L*
Endive *	*Cichorium endivia*
Head Lettuce *	*Lactuca sativa var. capitata*
Tarragon *	*Artemisia dracunculus L.*

Solanaceae Family

Common Name	Latin Name
Aubergine *	*Solanum melongena L*
Pepper *	*Capsicum annuum var. annuum L*
Potato	*Solanum tuberosum L*
Tomato	*Solanum esculentum L*

Cucurbitaceae Family

Common Name	Latin Name
Bitter Cucumber *	*Momordica charantia*
Chayote *	*Sechium edule*
Courgette	*Cucurbita pepo conv. giromontina*
Cucumber	*Cucumis sativus L.*
Marrow *	*Cucurbita pepo conv. Pepo*
Singkwa Towel Gourd *	*Luffa acutangula*
White gourd *	*Benincase hispida*
Winter Squash *	*Cucurbita moschata*

Apiaceae Family

Common Name	Latin Name
Carrot	*Daucus carota var. Sativus*
Celery	*Apium graveolens var. dulce*
Celeriac *	*Apium graveolens var. rapaceum*
Coriander	*Coriandrum sativum*
Dill *	*Anethum graveolens*
Fennel *	*Foeniculum vulgare*
Parsley *	*Petroselinum crispum var. crispum*
Parsnip *	*Pastinaca sativa*

Table 2.1: Sources Screened from Different Plant Families.

The sources purchased from commercial suppliers are indicated with *.

Lamiaceae Family

Common Name	Latin Name
Basil *	*Ocimum basilicum*
Mint *	*Mentha spicata*
Rosemary *	*Rosmarinus officinalis*
Sage *	*Salvia officinalis*
Thyme *	*Thymus vulgaris*

Poaceae Family

Common Name	Latin Name
Barley	*Hordeum vulgare subsp. Vulgare* var. Dandy
Rye	*Secale cereale*
Sorghum	*Sorgum bicolor*
Summer Wheat	*Triticum aestivum*
Winter Wheat	*Triticum aestivum*

Brassicaceae Family

Common Name	Latin Name
Broccoli	*Brassica oleracea var. italica*
Brussel Sprout	*Brassica oleracea var. gemmifera*
Cabbage (green)	*Brassica oleracea var. capitata*
Cabbage (white) *	*Brassica oleracea var. capitata*
Cauliflower	*Brassica oleracea var. botrytis*
Chinese Radish *	*Raphanus sativus var. niger*
Chinese Cabbage *	*Brassica pekinensis*
Indian Mustard *	*Brassica juncea*
Kale (green) *	*Brassica oleracea var. acephala*
Kale (red) *	*Brassica oleracea var. acephala*
Green Radish *	*Raphanus sativus L*
Red Radish	*Raphanus sativus L*
Swede	*Brassica napus var. napobrassica*
Turnip	*Brassica rapa var. rapa*

Fabaceae Family

Common Name	Latin Name
Scarlet Runner Bean *	*Phaseolus coccineus*
French Bean *	*Phaseolus vulgaris*
Bean Sprouts *	*Vigna radiata*
Broad Bean *	*Vicia faba*
Fenugreek *	*Trigonella foenum-graecum L*
Sugar Snap Pea	*Pisum sativum*
Yam Bean *	*Pachyrhizus erosus*

Table 2.1: Sources Screened from Different Plant Families.

The sources purchased from commercial suppliers are indicated with *.

All sources were rinsed using deionised water prior to the isolation of components by homogenisation and subcellular fractionation.

2.4 CULTIVATION OF *POACEAE* FAMILY SOURCES

'Dandy' barley, rye, sorghum, 'summer' and 'winter' wheat seedlings were grown using a range of cultivation methods. Prior to cultivation, all seeds were sterilised using 66 % (v/v) ethanol, and soaked for 5 hours in deionised water following Raquena and Bornemann's method [85].

2.4.1 Cultivation in Propagator

Poaceae seedlings were cultivated in a heated 30 cm by 42 cm sized propagator at approximately 25 °C on a mesh frame immersed in deionised water. The deionised water was replaced every two days and continuous aeration was maintained using air pumps.

2.4.2 Cultivation in Growing Chamber

Three growing chambers were used to cultivate seedlings from the *Poaceae* family in nine 30 cm by 42 cm sized trays. The seedlings were exposed to artificial illumination by tungsten lamps for 12 hours per day. Temperature was maintained at 25 °C and deionised water was replaced daily to ensure adequate aeration since air pumps were not used.

2.4.3 Other Cultivation Methods

Seedlings were also cultivated in the 'open atmosphere' in various growth medium: soil, gravel and silver sand in 30 cm by 42 cm sized trays. Seedlings were scattered on the surface of 2 inches of growth medium, after which they were covered with 1 cm of respective medium. All seedlings were watered when required dependent on weather conditions with deionised water.

All roots, shoots and seedlings of the crops cultivated using various methods were extracted after 12 days and analysed.

2.5 ISOLATION AND SUBCELLULAR FRACTIONATION OF OXALATE OXIDASE

Homogenisation during the screening of Oxox from possible sources was performed using a Braun hand blender in a 3:1 v/w ratio for 15 minutes in chilled deionised water. The resultant homogenate was filtered through a mesh (100-200 μm gauge) and centrifuged at

15,000 x g for 50 minutes at 4 °C. The supernatant after centrifugation was collected containing soluble components. The pellet were redissolved in 1/10th of the initial volume and treated with 10 % w/v sodium deoxycholate to release insoluble membrane bound components. The sodium deoxycholate treated components were stirred using a magnetic stirrer for 24 hours at 4 °C, after which they were centrifuged at 13,000 x g for 20 minutes at 4 °C. The resultant supernatant was collected containing released membrane components. The isolated soluble and released insoluble membrane components of each possible source screened were assayed using two types of assay mixtures, one was prepared with (+) oxalate, and the other was prepared in the absence (–) of oxalate.

2.5.1 Isolation of Oxalate Oxidase from the Poaceae Family

The sources from the *Poaceae* family: 'Dandy' barley, rye, sorghum, 'summer' and 'winter' wheat were homogenised using a pestle and mortar rather than a blender due to the small quantities of plant material cultivated. Subsequent centrifugation and treatment with sodium deoxycholate was performed as described in chapter 2.5.

Once possible sources of Oxox were isolated, subsequent homogenisation was more intense by shearing components with a PTFE Elvenhijm homogeniser.

2.6 THERMAL TREATMENT

The thermal stability of Oxox from the possible sources was determined by heating extracts for 10 minutes at 60 °C in a water bath. Thermally treated extracts were then centrifuged at 15,000 x g for 10 minutes at 4 °C removing precipitated components. The pellet component was discarded after centrifugation and the supernatant containing clarified thermally stable components was retained for further analysis. Assays were conducted prior to and after thermal stability studies using + and – oxalate assay mixtures.

2.6.1 Additional Thermal Stability Studies

The thermal stability of a selection of Oxox sources was investigated to determine the effect of elevated temperatures for prolonged durations. Extracts were heated at 100 °C for defined times of 10, 30 and 60 minutes in a water bath, the resultant extracts were

centrifuged at 13,000 x g for 10 minutes, after which the supernatant containing thermally stable components were retained.

2.7 AMMONIUM SULPHATE FRACTIONATION

Ammonium sulphate fractionation was performed in two stages, at an initial concentration of 35 %, followed by 65 %. Ground ammonium sulphate was added to the extracts according to the information in Data for Biochemical Research [137]. After the initial addition of 35 % ammonium sulphate, extracts were stirred for 20 minutes at 0 °C, after which they were centrifuged at 15,000 x g for 10 minutes at 4 °C. The supernatant was collected and used for the second stage of 65 % ammonium sulphate fractionation. Extracts were stirred for an additional 20 minutes at 0 °C and centrifuged further at 13,000 rpm for 10 minutes at 4 °C. The pellet containing precipitated proteins was retained and resuspended in $1/10^{th}$ of the initial volume and dialysed according to chapter 2.8.

2.8 DIALYSIS

Dialysis tubing was boiled in deionised water, soaked for 5 hours and rinsed with cold deionised water prior to the addition of the resuspended 65 % ammonium sulphate pellet sample. A knot was tied at the bottom of the tubing and clamped with a dialysis clip, after which the sample was added. The dialysis tubing was sealed and clamped at the other end with a dialysis clip to prevent leakage, forming a dialysis sack. The dialysis sack was attached to a float at one end and immersed in 100 times the volume of the sample and dialysed against appropriate buffer for 24 hours at 4 °C with continuous stirring. The appropriate dialysis buffer was determined by the subsequent purification procedure to be undertaken.

2.9 ION EXCHANGE CHROMATOGRAPHY

Cation and anion exchange chromatography were performed following differing methods. All buffers used were chilled, filtered and de-gassed prior to performing ion exchange chromatography.

2.9.1 Cation Exchange Chromatography

Carboxymethyl (CM) cellulose cation exchange media was used to purify possible Oxox. The cation exchange column was equilibrated using 10 mM acetate buffer pH 5.0, following this, the sample was applied to the chromatography column. Unbound components were eluted using this buffer, followed by the elution of bound components with a salt gradient of 1 M sodium chloride in 10 mM acetate buffer pH 5.0. All fractions collected were assayed for Oxox activity using + and – oxalate assay mixtures according to chapter 2.12.

2.9.1.1 Cation Exchange Chromatography of Sigma, Barley Root and Spanish Mint Leaves Oxalate Oxidase

Cation exchange chromatography of Sigma, barley root and Spanish mint Oxox was undertaken at room temperature at approximately 22 °C using the ÄKTA prime® system. The cation exchange media, CM cellulose was packed into a XK 2.6 cm x 14 cm column. 5 mL of the sample was applied to the column using a 5 mL injection loop. Unbound components were eluted using 100 mL 10 mM acetate buffer pH 5.0 and bound components were eluted using 200 mL salt gradient with 1 M sodium chloride in 10 mM acetate buffer pH 5.0 at 1 mL/minute flow rate. All eluted components were collected in 5 mL fractions and assayed for Oxox activity.

2.9.1.2 Cation Exchange Chromatography of Cabbage and Carrot Leaves Extracts

Cation exchange chromatography of cabbage and carrot leaves was performed at approximately 22 °C, the cation media was packed into an empty 2 cm x 7 cm Sigma Aldrich Ltd. PD10 column. 2.5 mL of extract was applied to the column manually, unbound components were eluted using 20 mL 10 mM acetate buffer pH 5.0. Bound components were eluted using a 0 to 1 M sodium chloride step gradient with increments of 0.25 M sodium chloride (20 mL) in 10 mM acetate buffer pH 5.0. All components were eluted under gravity and 0.5 mL fractions were collected and analysed for Oxox activity.

2.9.2 Anion Exchange Chromatography

The anion exchange media diethylaminoethyl (DEAE) cellulose was packed into an empty 2 cm x 7 cm Sigma Aldrich Ltd. PD10 column. All anion exchange chromatography was

undertaken at approximately 22 °C under gravity. The anion exchange column was equilibrated using 50 mL 20 mM Tris pH 8.5 and 2.5 mL of sample was applied to the column manually. Unbound components were eluted using the same buffer, however, bound components were eluted using 1 M sodium chloride in 20 mM Tris pH 5.0, in increments of 0.2 M (15 mL). All eluted components were collected in 0.5 mL aliquots and assayed for Oxox activity using both + and – oxalate assay mixtures following method 2.12.

2.10 AFFINITY CHROMATOGRAPHY

The affinity chromatography media employed to purify Oxox was Concanavalin A-Sepharose 4B which was packed into a XK 2.6 cm x 15 cm column. Affinity chromatography was performed using the ÄKTA prime® system at approximately 22 °C using chilled, filtered and de-gassed buffers. The affinity column was equilibrated using 150 mL 50 mM acetate buffer pH 5.0. 2 mL of the extract was applied to the column using a 2 mL injection loop, unbound components were eluted using 150 mL 50 mM acetate buffer pH 5.0. Bound components were eluted using 150 mL 2 mM methyl-α-D-mannopyroside prepared in 50 mM sodium acetate buffer pH 5.0. Following this, 10 mL 1 M sodium hydroxide in 50 mM acetate buffer pH 5.0 was applied to the column for sanitisation purposes. All samples eluted from the column were assayed for Oxox activity, using + and – oxalate assay mixtures. The flow rate was set at 1 mL/min and 5 mL fractions were collected.

2.11 GEL FILTRATION CHROMATOGRAPHY

Sephacryl S-200-HR, Sephadex G25, G15, G10 and Bio Gel P2 media were used to purify Oxox. Most gel filtration chromatography was performed using the ÄKTA FPLC system at 4 °C and all buffers were filtered, chilled and degassed prior to use. Eluted components were assayed using both + and – oxalate assay mixtures according to chapter 2.12.

2.11.1 Calibration of the Sephacryl S-200-HR Column

Sephacryl S-200-HR media was packed into a 2.6 cm x 76 cm XK column and equilibrated using 50 mM potassium phosphate pH 7.0 with the flow rate set at 0.1 mL/min. The

Sephacryl S-200-HR column was calibrated using a number gel filtration molecular weight markers ranging from 12.4 kDa to 200 kDa, listed below:

- Cytochrome c 12.4 kDa
- Carbonic anhydrase 29 kDa
- Bovine serum albumin 66 kDa
- Alcohol dehydrogenase 150 kDa
- β amylase 200 kDa

2 mL of the standard molecular marker mixture was applied to the Sephacryl S-200-HR column, using a 2 mL injection loop. Figure 2.1 illustrates the resultant chromatograph:

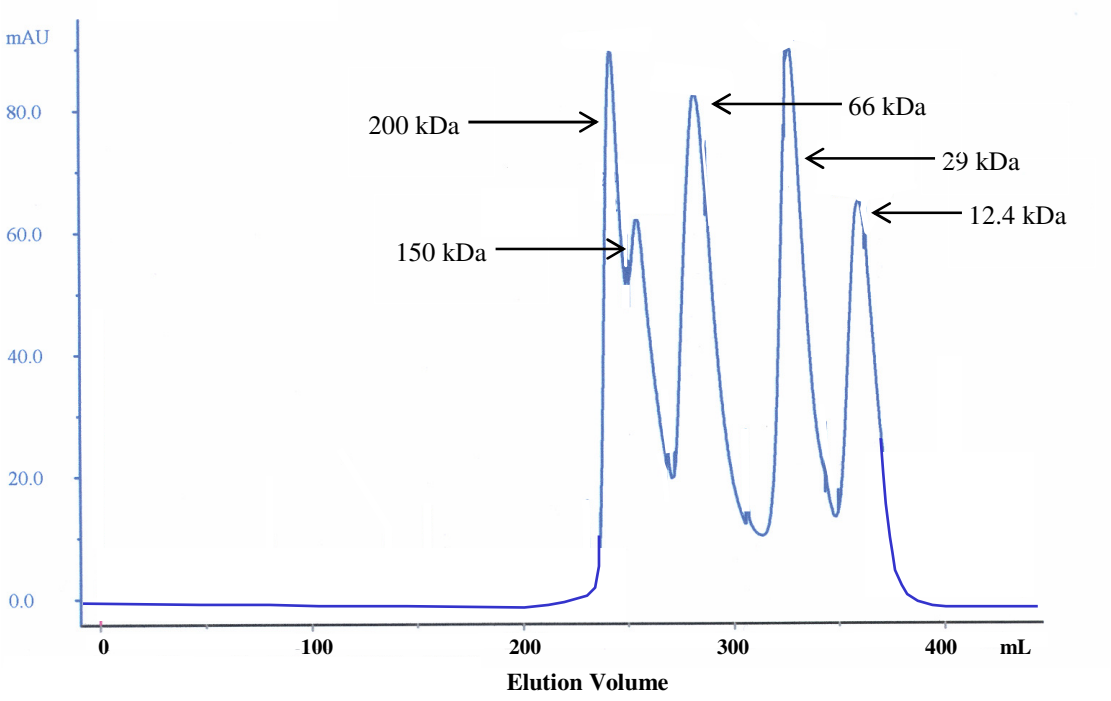


Figure 2.1: Calibration of the Sephacryl S-200-HR Column using Molecular Mass Protein Standards.

Absorbance at 280 nm was measured with an on-line spectrophotometric detector.

The calibration of the Sephacryl S-200-HR column was essential as it would aid the interpretation of the approximate molecular mass of the components eluted from the column during the purification studies.

2.11.1.1 Gel Filtration Chromatography of Cabbage Leaves Oxalate Oxidase Using Sephacryl S-200-HR

2 mL extracts were applied to the calibrated Sephacryl S-200-HR column using a 2 mL injection loop. Components were eluted from the column with 1.2 L 50 mM potassium phosphate pH 7.0 containing 0.1 M sodium chloride with the flow rate at 0.1 mL/min for 24 hours and 10 mL fractions were collected.

2.11.2 Gel Filtration Chromatography of Barley Root Oxalate Oxidase using Sephacryl S-200-HR

Sephacryl S-200-HR media was packed into a 2.6 cm x 44 cm XK column. Gel filtration chromatography was performed at 22 °C using the ÄKTA prime® system. 2 mL sample was applied to the column using a 2 mL injection loop. Components were eluted using 100 mM 50 mM potassium phosphate pH 7.0 at 0.5 mL/min flow rate, with 5 mL fractions being collected.

2.11.3 Calibration of Sephadex G25, G15 and G10 Columns

Sephadex G25 XK 2.6 cm x 42 cm, and Sephadex G15 and G10 2.6 cm x 11 cm XK columns were used during gel filtration chromatography. The Sephadex columns were equilibrated with deionised water with the flow rate set at 0.5 mL/min.

Sephadex G25, G15 and G10 columns were standardised using bovine serum albumin (BSA) containing 1 M sodium chloride. 40 mL of this preparation was applied to the Sephadex G25 column using a super loop. This standardisation procedure was undertaken to illustrate the 'group separation' using Sephadex G25 media, since proteins with a molecular mass of more than 5 kDa elute in the initial 'protein peak' followed by the elution of smaller molecular mass components, less than 5 kDa, in the second peak. Figure 2.2 demonstrates the separation achieved after the chromatography of BSA and sodium chloride using Sephadex G25 chromatography. Protein and salt elutions were monitored by online spectrophotometric and conductometric measurements respectively.

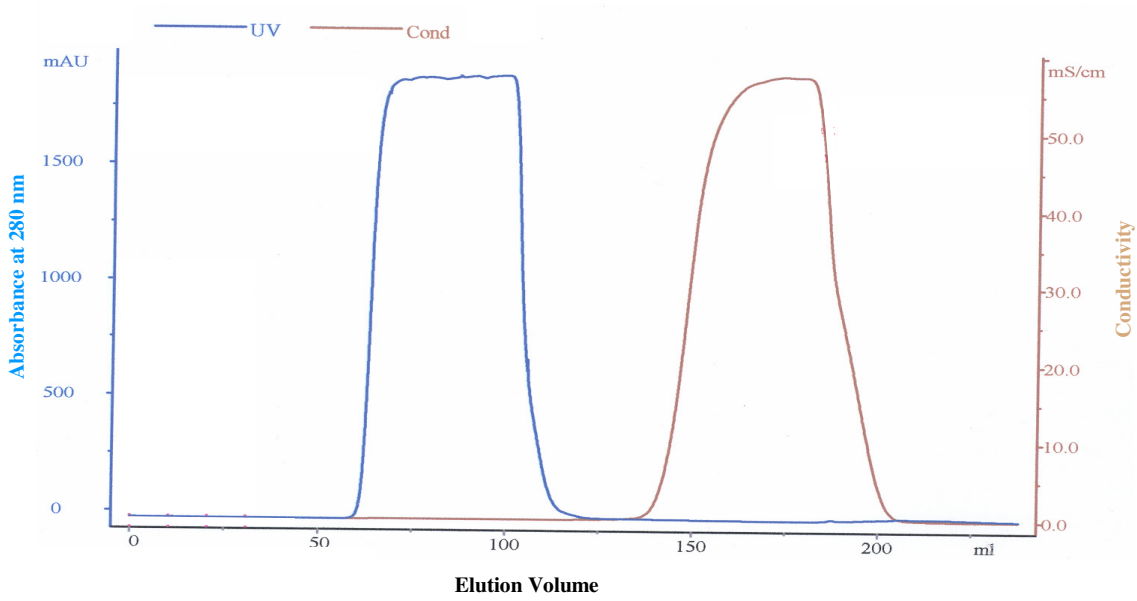


Figure 2.2: The Separation of Bovine Serum Albumin and Sodium Chloride using Sephadex G25 Chromatography.

Conductivity and A_{280nm} were monitored online.

The separation of the salt component (sodium chloride) from the protein component (BSA) was evident after performing Sephadex G25 chromatography. Similar calibration experiments were undertaken with Sephadex G15 and G10 columns. The separation of BSA from sodium chloride was clearly visible during calibration studies (results not shown).

2.11.4 Gel Filtration Chromatography using Sephadex G25, G15 and G10

The calibrated and standardised Sephadex G25, G15 and G10 columns were used to resolve the components of interest in plant extracts. 40 mL extracts were applied to the Sephadex G25 column, however 3 mL extracts were applied to the Sephadex G15 and G10 columns. Components were eluted at a flow rate at 0.5 mL/min using deionised water.

2.11.5 Gel Filtration Chromatography using Bio Gel P2

Bio Gel P2 XK column (2.6 cm x 44 cm) was equilibrated using deionised water. 5 mL extracts were applied to the Bio Gel P2 column using a 5 mL injection loop. Components were eluted using deionised water at 0.5 mL/min flow rate with the collection of 1 mL fractions.

2.12 ENZYMATIC ASSAYS

Prior to initiating purification procedures, it was imperative to ascertain which of the many available enzymatic assays were most stable, specific and reproducible for Oxox. An important requisite of an enzymatic assay is the stability of the assay mixture ensuring it does not deteriorate over short periods of time leading to 'false positive' Oxox activities. The possible interactions between the assay mixture and the assayable species may also further yield false positive results.

During the purification of Oxox from alternative sources, the procedures employed may dilute the concentration of Oxox, in turn yielding lower Oxox activities. To ensure low rates are detected, the enzymatic assay used must possess low limits of detection.

A number of coupled peroxidase catalysed enzymatic assays have been reported to detect the presence of Oxox, discussed in chapter 1.2.2. Hydrogen peroxide generated by Oxox reacts with the dye precursor(s) catalysed by horseradish peroxidase (HRP) to yield a coloured dye. The change in the intensity of the coloured dye at an appropriate wavelength is a representation of the rate of oxalate oxidised by Oxox, and is proportional to the hydrogen peroxide formed in equimolar amounts to oxalate being oxidised.

Four well established enzymatic assays are employed by research groups: ABTS, aminophenazone, σ-diansidine and the MBTH assays, however, the concentration(s) of the dye precursor(s) within the enzymatic assay do not alter. As a result, studies were conducted to determine if the concentration(s) reported were in fact adequate to interact with the hydrogen peroxide generated. To investigate this, concentration profiles were constructed for the dye precursor(s) within each of the four assays to ensure saturation was achieved.

Concentration profiles were conducted using a related enzyme, glucose oxidase (Gox) due to the high cost of Sigma Oxox. Gox and Oxox are similar in that both produce hydrogen peroxide as the co-product of the coupled enzyme assay. The hydrogen peroxide then reacts with the dye precursor(s) and horseradish peroxidase (HRP) yielding a coloured dye.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} H_2O_2 + \text{Gluconic Acid}$$

$$H_2O_2 + \text{Dye Precursor(s)} \xrightarrow{\text{Horseradish Peroxidase}} \text{Coloured Dye} + H_2O$$

Concentration profiles were constructed for each dye precursor in the ABTS, aminophenazone, σ-diansidine and MBTH assays. The concentrations of Gox, HRP and glucose remained constant throughout these studies, eliminating potential variables.

2.12.1 MBTH Assay

The MBTH assay employs a combination of two dye precursors: N,N-dimethylaniline (DMA) and 3-methyl-2-benzothiazolinone hydrazone (MBTH). The MBTH assay reaction system is composed of the following components at final concentrations of:

Assay mixture:
- 0.1 M Phosphate buffer pH 5.5
- 3.5 mM MBTH
- 25.9 mM DMA
- 30 mM Glucose
- 2.5 U Horseradish peroxidase
- 2.4 U Glucose oxidase

Reactions were initiated by the addition of HRP and the enzyme simultaneously to the assay mixture containing the dye precursors and glucose. Concentration profiles of the dye precursors were constructed by altering the concentration of one dye precursor of interest and keeping the other constant. DMA and MBTH concentration profiles were performed ranging from 15 mM to 40 mM and 2.5 mM to 5.5 mM respectively. The rate of Oxox activity was measured at 578 nm and each assay was performed in triplicate. The results obtained after constructing concentration profiles are shown in figure 2.3a (DMA) and figure 2.3b (MBTH).

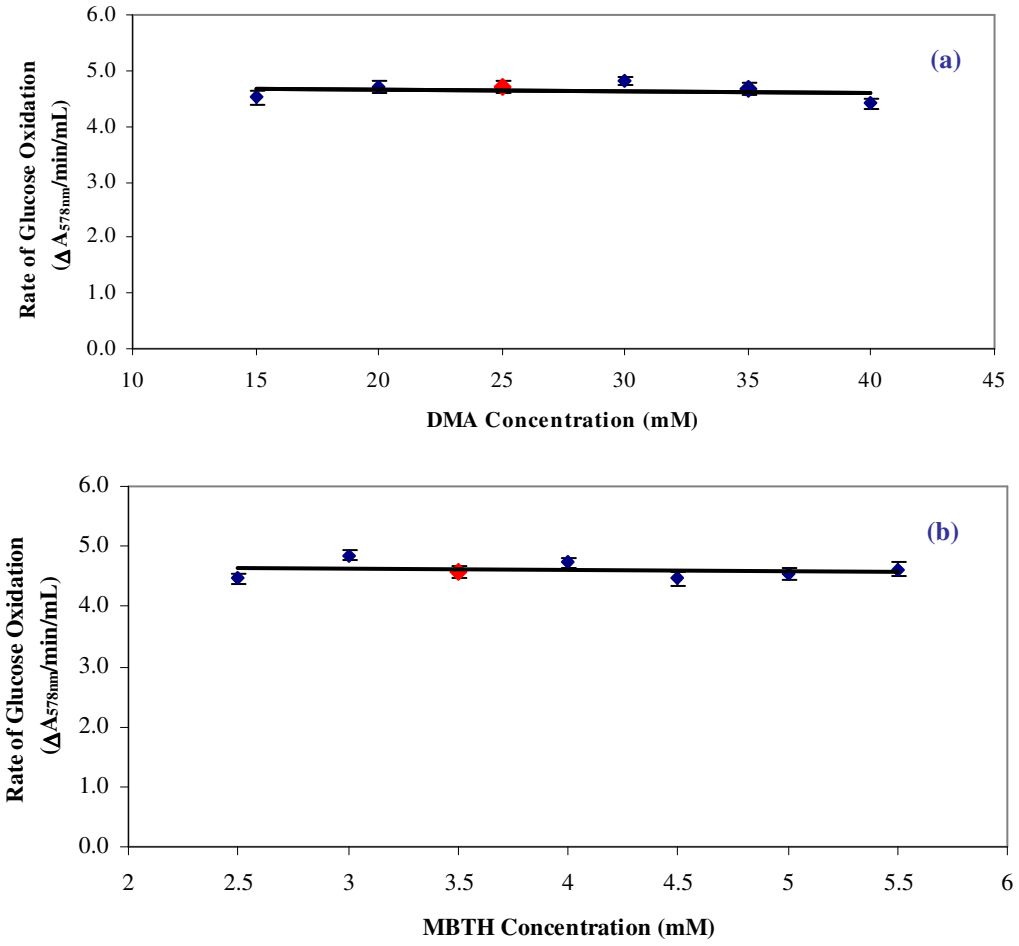


Figure 2.3: The Effect of DMA and MBTH Concentration on the MBTH Assay.

The concentration of DMA (a) and MBTH (b) used in a conventional assay is denoted by ♦. The rate of glucose oxidation was monitored at ΔA_{578nm} and all enzymatic assays were performed in triplicate. Error bars were calculated by standard deviation, N=3.

Increasing the concentrations of either DMA or MBTH showed no significant increase in sensitivity with regards to the measured rate of glucose oxidation. Consequently, the profiles constructed demonstrated that the concentrations stated in the literature of 25.9 mM of DMA and 3.5 mM for MBTH were adequate to react with the hydrogen peroxide produced to yield the indamine dye.

2.12.2 Aminophenazone Assay

The aminophenazone assay also involves the use of two dye precursors: 4-aminophenazone (aminophenazone) and phenol. The final concentration of the components in the reaction system of the aminophenazone assay are:

Assay mixture: 0.1 M Phosphate buffer pH 5.5
0.33 mM Aminophenazone
3.3 mM Phenol
30 mM Glucose
2.5 U Horseradish peroxidase
2.4 U Glucose oxidase

Reactions were initiated by the addition of HRP and the enzyme simultaneously to the assay mixture containing the dye precursors and the substrate, glucose. The rate of oxidation was measured at 520 nm and each assay was performed in triplicate. Concentration profiles were constructed for both aminophenazone and phenol ranging from 0.25 mM to 0.55 mM and 2.5 mM to 5.5 mM respectively. The results are shown in figure 2.4a (aminophenazone) and figure 2.4b (phenol).

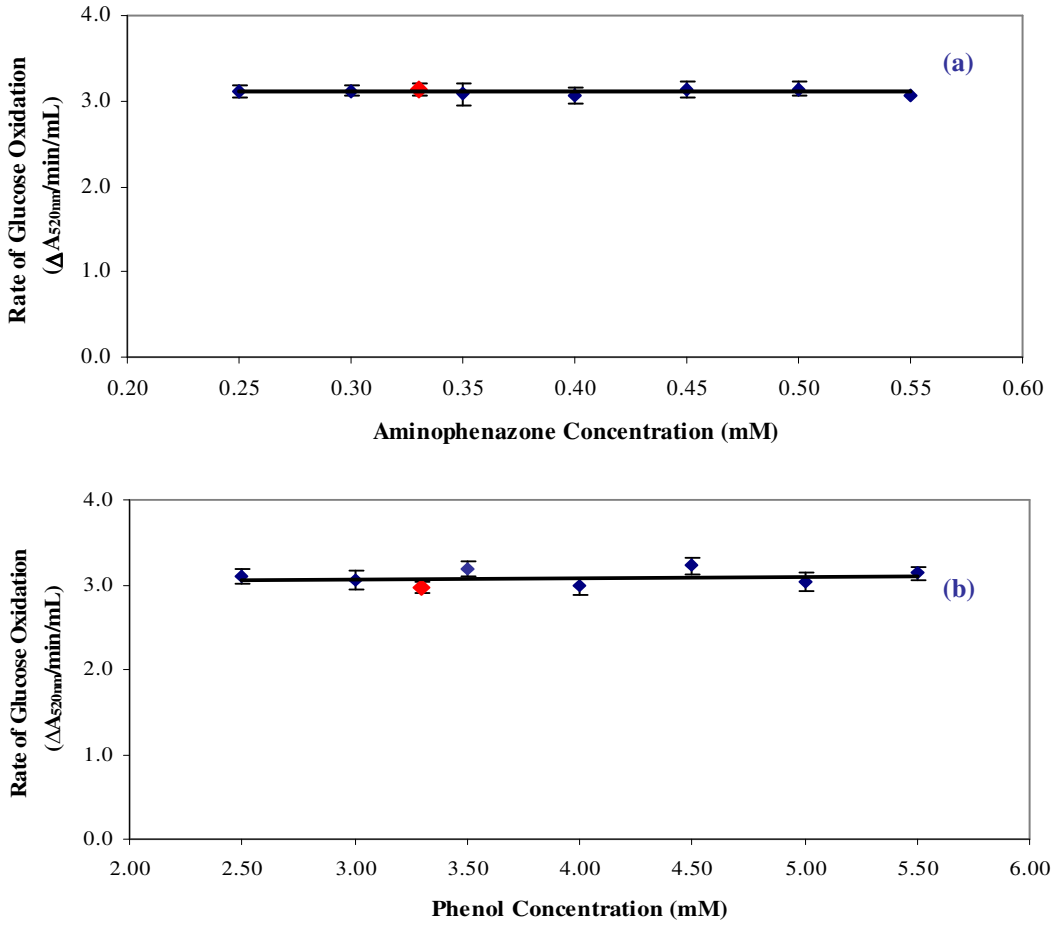


Figure 2.4: The Effect of Aminophenazone and Phenol Concentration on the Aminophenazone Assay.

The concentration of aminophenazone (a) and phenol (b) used in a standard assay is denoted by ♦. The rate of glucose oxidation was monitored at ΔA_{520nm}, all assays were performed in triplicate. Error bars were calculated by standard deviation, N=3.

The concentration profiles of aminophenazone and phenol led to the finding that increasing the concentration of either dye precursor did not significantly alter the sensitivity of the assay. As a result, these studies demonstrate the concentrations of aminophenazone and phenol in the literature of 0.33 mM and 3.3 mM respectively are sufficient.

2.12.3 ABTS Assay

The ABTS assay comprises only one dye precursor: 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS), and is used in a standard assay at the concentration of 1.82 mM. ABTS assays were performed using the following components in the reaction system at the final concentrations of:

Assay mixture: 0.1 M Phosphate buffer pH 5.5
1.82 mM ABTS
30 mM Glucose
2.5 U Horseradish peroxidase
2.4 U Glucose oxidase

Enzymatic assays were initiated by the addition of HRP and Gox simultaneously to the assay mixture containing glucose and ABTS. The rate was measured at 670 nm and each assay was performed in triplicate. To investigate if the concentration of ABTS was sufficient to interact with hydrogen peroxide and HRP in the coupled enzymatic assay, a concentration profile was constructed ranging from 1.0 mM to 3.5 mM, the results of which are shown in figure 2.5.

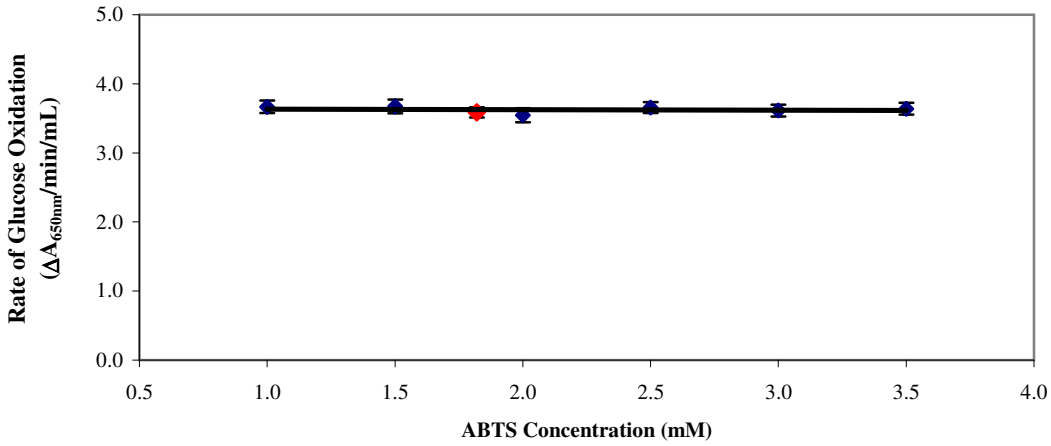


Figure 2.5: The Effect of ABTS Concentration on the ABTS Assay.

The concentration of ABTS used in a standard assay is denoted by ♦. The rate of glucose oxidation was monitored at ΔA_{650nm} and all assays were performed in triplicate. Error bars were calculated by standard deviation, N=3.

Altering the concentration of the dye precursor did not lead to significant changes in the sensitivity of the assay. Therefore, it can be concluded the conventionally used ABTS concentration of 1.82 mM is in fact adequate.

2.12.4 σ-Diansidine Assay

The concentration of σ-diansidine in a standard assay is 0.21 mM. The σ-diansidine assay employs only one dye precursor: σ-diansidine and the reaction system is composed of:

Assay mixture:
0.1 M Phosphate buffer pH 5.5
0.21 mM σ-Diansidine
30 mM Glucose
2.5U Horseradish peroxidase
2.4U Glucose oxidase

The rate of glucose oxidation was monitored at 500 nm, reactions were initiated by the addition of HRP and the enzyme simultaneously to the assay mixture and each assay was carried out in triplicate. A concentration profile ranging from 0.05 mM to 2 mM was constructed to determine if the concentration of σ-diansidine was adequate in the reaction system to yield a coloured dye.

In comparison to the previous three assays, the concentration profile for σ-diansidine proved to be problematic. The major difficulty encountered during the construction of the σ-diansidine concentration profile was the instability of the assay mixture over a short period of 30 minutes. The addition of Gox to the reaction system initially resulted in a sharp increase in the absorbance change, followed by a decline (figure 2.6a), this was contrary to an expected gradual increase (figure 2.6b). A comparison of the rate measured spectrophotometricaly using the σ-diansidine and MBTH assays are shown in figure 2.6a and figure 2.6b respectively.

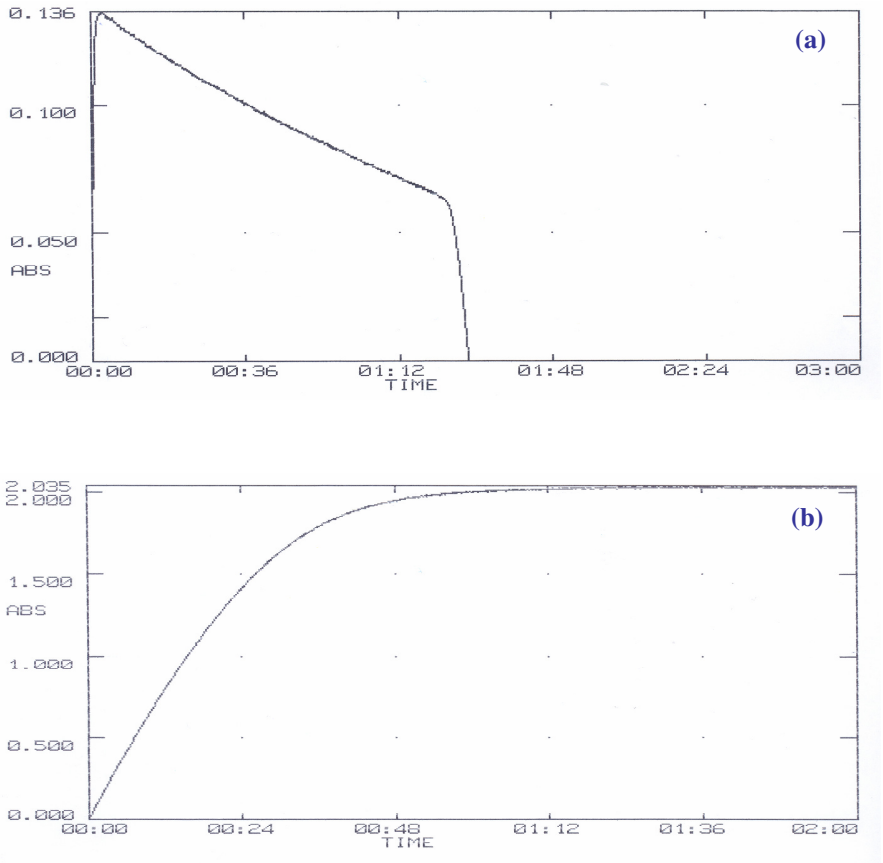


Figure 2.6: Spectrophotometric Measurements of Glucose Oxidase Using the σ-Diansidine and MBTH Assays.

Enzymatic assays were carried out using the σ-diansidine assay (a) at A_{500nm} and MBTH assay (b) at A_{578nm}.

In addition to the unconventional spectrophotometric measurements, false positive rates of reaction were also generated using the σ-diansidine assay since the coloured dye was generated in the absence of Gox. The dissolution of σ-diansidine in the assay buffer was

also problematic, unlike that of the ABTS, aminophenazone and MBTH assays where the dye precursor(s) dissolved instantaneously.

2.12.5 Overview of Assay Selection

Each enzymatic assay was studied to determine the effect of assaying with reduced or excessive concentrations of the dye precursor(s) within the ABTS, aminophenazone, σ-diansidine and MBTH assays.

The instability of the σ-diansidine assay mixture, the problems with its dissolution coupled with its toxic and carcinogenic properties led to the elimination of this assay for use during this research. The remaining three colorimetric assays: ABTS, aminophenazone and MBTH assays were reliable and reproducible detecting the hydrogen peroxide generated by the oxidation of glucose by Gox.

The aminophenazone assay employs phenol as a dye precursor within the enzymatic assay, since some reported Oxox sources contain polyphenol oxidase (PPO) [71], the use of this assay would be selective depending on the source investigated. The presence of PPO in reported sources may generate hydrogen peroxide by phenol reacting with oxygen in the reaction system.

The ABTS and the MBTH assays were more sensitive than the aminophenazone assay, however, all three assays generated reproducible, repeatable and reliable activities. In conclusion, the ABTS, aminophenazone and MBTH assays will be employed to detect oxalate oxidation by Oxox in possible sources.

2.12.6 Assay for Oxalate Oxidase

All Oxox enzymatic assays performed during this research contained the components listed in table 2.2 in the reaction system to measure Oxox present in the sources screened.

Aminophenazone Assay	ABTS Assay	MBTH Assay
3.3 mM Phenol	1.82 mM ABTS	3.5 mM MBTH
0.33 mM Aminophenazone		25.9 mM DMA
45 mM Succinate buffer pH 3.8		
0.1 U to 0.2 U Oxalate oxidase/10 µL to 500 µL extract		
2.5 U Horseradish peroxidase		
1.31 mM Oxalate		

Table 2.2: Components Present within the Aminophenazone, ABTS and MBTH Assays.
The colour reagents of each enzymatic assay are indicated in red.

Assays were initiated by the addition of HRP and the extract containing Oxox simultaneously, all assays were performed in triplicate. The rates of oxalate oxidation were monitored at 650 nm, 520 nm and 578 nm for the ABTS, aminophenazone and MBTH assays respectively.

Assay mixtures were prepared containing (+) the substrate oxalate, and in the absence (-) of the substrate to determine if the activity in the reaction system was "real oxalate oxidase activity". Oxalate – assay mixtures were prepared in a similar manner to those in table 2.2, however, in the absence of oxalate.

2.13 BICINCHONINIC ACID PROTEIN ASSAY

Bicinchoninic acid (BCA) protein assay reagents were used to detect protein content in purified extracts following Sigma Aldrich Ltd. protocol BCA-1 and B 9643 during this research. BCA protein assay possesses a detection range of 20 µg/mL to 2000 µg/mL and detect proteins by the presence of cysteine, cystine, tryptophan, tyrosine and peptide bonds [138].

2.14 SODIUM DODECYL SUPHATE POLYACRYLAMIDE GELS

ELECTROPHORESIS

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) is a method of separating polypeptides according to their molecular mass. Protein electrophoresis involves

the use of SDS and dithiothreitol (DTT) which denature and cleave disulphide bonds, leading to the total unfolding of proteins.

10 % Bis-Tris polyacrylamide gels were used and prepared following Invitrogen's protocol booklet N4458. The loaded PAGE gels were run at 200 V constant for 30 minutes in NuPAGE® SDS Running Buffer, after which they were stained following methods 2.15 and 2.16.

2.15 COOMASSIE BLUE STAINING OF POLYACRYLAMIDE GELS

Brilliant Blue R250, also known as Coomassie Brilliant Blue, was used as a stain to visualise proteins on polyacrylamide gels. Coomassie Blue binds non-specifically to almost all proteins and has the detection limit of 0.1 μg to 0.5 μg of protein [139].

Coomassie Blue stain was prepared by dissolving 0.25 % v/v Brilliant Blue R250 in 7 % v/v acetic acid. Destain was prepared using 10 % v/v acetic acid and 30 % v/v methanol in deionised water.

2.16 SILVER NITRATE STAINING OF POLYACRYLAMIDE GELS

Silver nitrate staining aided the visualisation of proteins on polyacrylamide gels and possesses a detection limit of 0.2 ng to 0.6 ng [139]. Silver nitrate staining was a multi-step process, whereby a number of reagents were employed including, ethanol, acetic acid, glutaraldehyde 1.2 % v/v, formaldehyde 0.19 % v/v, sodium thiosulphate 0.5 % v/v, silver nitrate 2.5 % v/v, sodium carbonate and glycerol. Fluka silver staining protocol 85181 was followed to stain polyacrylamide gels.

2.17 MASS SPECTROMETRY

All samples submitted for mass spectrometry were analysed 'in house' by technical staff. The samples were analysed in electrospray positive mode using a cone voltage of 40 V. Aliquots (10 μL) were injected into a 50/50 v/v methanol/deionised water mobile phase using a 20 μL loop. Product ion scans were acquired using a collision energy of 50 eV

using argon as collision gas. The same conditions were used for acquisition of precursor ion scans.

2.18 AMPEROMETRIC ANALYSIS

Amperometric analysis employed a three electrode format consisting of a platinum working electrode with a diameter of 1.6 mm, a counter electrode, and an Ag/AgCl reference electrode. The oxidation of oxalate was detected by measuring the production of hydrogen peroxide at a potential of 0.65 V versus Ag/AgCl.

All platinum electrodes were thoroughly polished using aluminium oxide (0.015 µm) prior to use and rinsed with deionised water. Electrodes were prepared using enzyme preparations with STY1 emulsion polymer (10 % solids), according to Mahmood [56] which consisted of 40 % styrene and 60 % n-butyl acrylate. The prepared electrodes were dried at 50 °C for 60 minutes, after which they were allowed to cool and rinsed with distilled water.

Amperometric analysis with Gox was carried out by preparing the enzyme in STY1 emulsion in concentrations of 4.0, 6.0 and 8.0 U/µL, however, Oxox was prepared with 1.5 U/µL only. 1 µL of mixtures were applied onto the inverted platinum stud electrode, shown in figure 2.7.

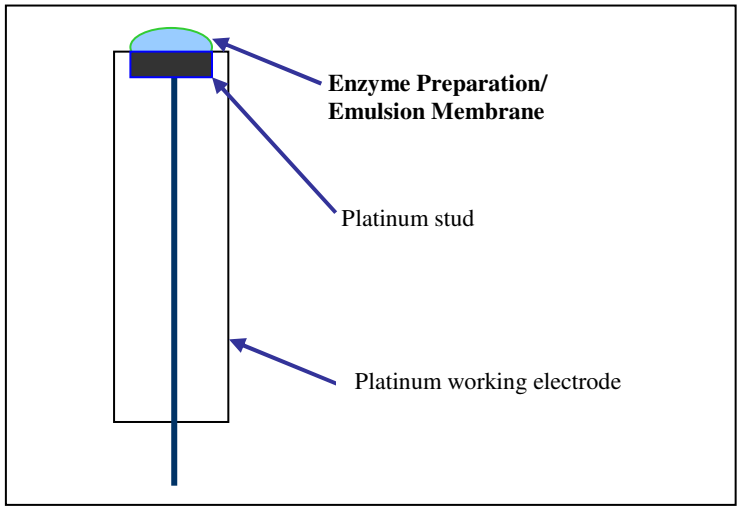


Figure 2.7: The Immobilisation of Enzyme Preparations onto a Platinum Electrode.

Oxox and Gox were immobilised onto a 3 mm platinum stud using STY1 emulsion polymer.

Amperometric studies were recorded by measuring the current response to sequential

additions of 1 mM glucose/ 0.1 mM oxalate over time. The additions were continued until the current response did not increase further and saturation was achieved. Each electrode measurement was performed in triplicate to assess the robustness of the biosensor.

CHAPTER 3

CHARACTERISATION OF SIGMA OXALATE OXIDASE

Oxalate oxidase (Oxox) is commercially available from Sigma Aldrich Ltd. and was also obtainable from Boehringer Mannheim until it was withdrawn in 1998. Sigma Oxox is purified from barley roots and has been reported to be insoluble, relatively impure (75 %) and varies in activity from batch to batch [138], posing questions regarding its quality.

Sigma Oxox was investigated in order to characterise the enzyme and gain an understanding of its properties. Optimum enzymatic assays, stability within various buffers, optimum buffer, effect of pH and substrate specificity of Sigma Oxox were investigated, since these factors are important attributes of any enzyme and directly affect the measured activity. The relatively impure commercial enzyme was purified employing a combination of purification procedures to establish an optimised protocol, which would be implemented to purify Oxox from alternative sources.

3.1 OPTIMUM ENZYMATIC ASSAY

The enzymatic assays performed in chapter 2.12 using glucose oxidase (Gox) demonstrated that the ABTS, aminophenazone and MBTH assays were reliable, repeatable and reproducible. Similar assay studies were conducted with Sigma Oxox in place of Gox. Enzymatic assays were performed using ABTS, aminophenazone and MBTH assay mixtures prepared in the presence (+) and in the absence (−) of oxalate, the results of which are shown in table 3.1.

	Oxalate Oxidase Activity (ΔA/min/mL)	
	+ Oxalate	**- Oxalate**
ABTS	1.846 ± 0.016	ND
Aminophenazone	1.306 ± 0.022	ND
MBTH	2.195 ± 0.012	ND

Table 3.1: Activity of Sigma Oxalate Oxidase using Different Enzymatic Assays.

0.1 U Oxox was assayed and activity was monitored at A_{650nm}, A_{520nm} and A_{578nm}, for the ABTS, aminophenazone and MBTH assays respectively. ND denotes 'none detected' and all enzymatic assays were performed in triplicate following method 2.12.6.

The Oxox activities measured using ABTS, aminophenazone and MBTH assays showed similar trends to those generated with Gox, with regards to the sensitivity of each assay. The ABTS, aminophenazone and MBTH assays were found to be reliable, reproducible and repeatable, however, significant variations in sensitivity were observed. The MBTH assay was the most sensitive, with the highest change in absorbance per minute being

measured in addition to the lowest standard deviation. As a result, the MBTH assay was used during the characterisation and purification of Sigma Oxox.

3.2 OPTIMUM BUFFER OF SIGMA OXALATE OXIDASE

Three main buffers are conventionally employed to purify Oxox, for instance, 10 mM to 50 mM sodium acetate pH 5 [76, 85, 114], 10 mM to 50 mM potassium/sodium phosphate pH 6.7 to 7.5 [72, 74, 75, 77, 82] and 20 mM to 50 mM sodium succinate pH 3.5 to 5 [75-77, 79, 83, 126]. Studies were performed to determine the optimum buffer and pH of Sigma Oxox. The buffers selected for these studies were: 50 mM sodium phosphate pH 7.0, 10 mM sodium acetate pH 5.0 and 45 mM sodium succinate pH 3.8. Once an optimum buffer and pH were established, further studies were undertaken to determine the buffering capacity of the buffer.

3.2.1 Optimum Buffer of Sigma Oxalate Oxidase

The purification of Oxox will employ 50 mM sodium phosphate pH 7.0, 10 mM sodium acetate pH 5.0 and 45 mM succinate pH 3.8, therefore, studies were performed to determine the optimum buffer for enzymatic assays. Conventionally, the fractions collected during purification procedures are analysed with assay mixture prepared using 45 mM sodium succinate pH 3.8 irrespective of the buffer used during subsequent procedures. These studies were undertaken to determine if the buffer used during purification could be used to assay the eluted fractions. For example, if cation exchange chromatography was performed using 10 mM sodium acetate pH 5.0, the assay mixture was prepared using the same buffer rather than 45 mM sodium succinate pH 3.8. The remaining assay conditions were maintained, with regards to the concentration of oxalate, Oxox, horseradish peroxidase (HRP) and dye precursors (DMA and MBTH). The resultant Oxox activities after preparing assay mixtures and Oxox with the three buffers are shown in table 3.2.

Buffer	Oxalate Oxidase Activity (ΔA_{578nm}/min/mL)
10 mM Sodium Acetate pH 5.0	1.624 ± 0.024
50 mM Sodium Phosphate pH 7.0	0.565 ± 0.021
45 mM Sodium Succinate pH 3.8	2.216 ± 0.023

Table 3.2: The Activity of Sigma Oxalate Oxidase in Different Buffers.

0.1 U Oxox was assayed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate.

The Oxox activity measured after preparing the assay mixture and Oxox in 45 mM sodium succinate pH 3.8 was approximately 25 % greater than the rate observed using 10 mM sodium acetate pH 5.0. Oxox activity was reduced by 75 % after preparing Oxox and assay mixtures using 50 mM sodium phosphate pH 7.0 in comparison to 45 mM sodium succinate pH 3.8. These findings demonstrated that Oxox activity was sensitive to the nature and pH of the assay buffers.

3.2.2 Optimum pH of 45 mM Sodium Succinate

Most enzymes possess a distinctive pH profile allowing the enzyme to be more stable and active. Fluctuations in pH may lead to a reduction, or conversely an increase in activity, illustrating the importance of pH. A pH profile for 45 mM sodium succinate was constructed to determine the optimum pH to ensure the Oxox activity observed during these assays was the maximum obtainable.

All assay conditions and components remained unchanged during these studies with regards to the concentration of the dye precursors, oxalate, Oxox and HRP, with the exception of the pH of 45 mM sodium succinate, the results of which are shown in figure 3.1.

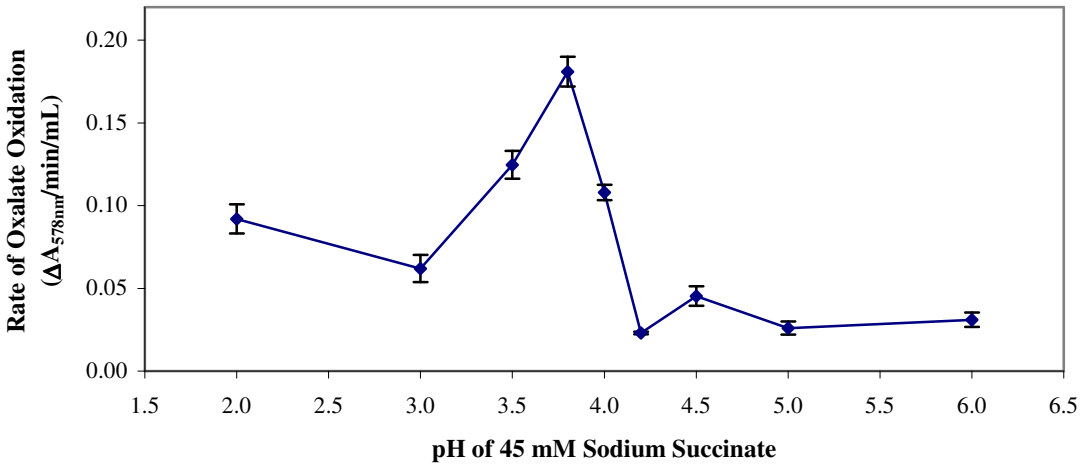


Figure 3.1: The pH Profile of Sigma Oxalate Oxidase in 45 mM Sodium Succinate.
Assays were performed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate. Error bars are a representation of standard deviation.

The optimum pH of 45 mM sodium succinate was found to be pH 3.8 where maximum Oxox activity was observed, deviations in pH 3.8 showed a decrease in Oxox activity.

3.2.3 The Buffering Capacity of 45 mM Sodium Succinate pH 3.8

During the purification of Oxox, the concentration of Oxox assayed may vary in the fractions collected. If low Oxox activities are expected, the volume of the fraction assayed would need to increase to achieve detectable activities. Having established pH 3.8 as the optimum pH, further studies were conducted to determine if the pH of the total assay mixture would alter by adding increased volumes of the assayable fraction (5, 10, 15 and 20 % v/v), present at a different pH, e.g. 50 mM sodium phosphate pH 7.0 or 10 mM sodium acetate pH 5.0. The pH of the total assay mixture was monitored prior to and after the addition of each buffer.

The addition of 5 % and 10 % v/v of either 50 mM sodium phosphate pH 7.0, or 10 mM sodium acetate pH 5.0 to 45 mM sodium succinate pH 3.8 did not alter the pH of the overall assay mixture. However, the addition of 15 % and 20 % v/v of 50 mM sodium phosphate pH 7.0 led to small but insignificant changes in the pH of 45 mM sodium succinate. Similar results were obtained after the addition of 15 % and 20 % v/v 10 mM sodium acetate pH 5.0, where the pH of 45 mM sodium succinate increased slightly.

3.2.4 Overview of the Optimum Buffer for Sigma Oxalate Oxidase

It is clearly evident 50 mM sodium phosphate pH 7.0 and 10 mM sodium acetate pH 5.0 are not suitable buffers for preparing Sigma Oxox and assay mixtures since a reduction in activity was observed in comparison to 45 mM sodium succinate pH 3.8. Further studies with the optimum buffer: 45 mM sodium succinate were undertaken to determine the optimum pH. The construction of a pH profile led to the confirmation that pH 3.8 was in fact the most favourable pH. These findings confirm the data available from Sigma, who recommend the use of 45 mM sodium succinate pH 3.8 with their commercially available oxalate assay kits. Oxox purified from a number of sources also possess similar optimum pH optima ranging from 3.5 to 4 [74-76, 78, 79, 82, 83, 97, 103, 104, 122, 125]. Consequently, 45 mM sodium succinate pH 3.8 was used throughout this research to prepare assay mixtures ensuring optimum Oxox activity was obtained.

The addition of varying volumes of up to 20 % v/v 50 mM sodium phosphate pH 7.0 and 10 mM sodium acetate pH 5.0 to 45 mM sodium succinate pH 3.8 was investigated to determine the buffering capacity of succinate buffer. These studies led to the finding that the change in the overall pH of the assay buffer (45 mM sodium succinate pH 3.8) was

minimal, therefore, increased volumes of the assayable fraction up to 20 % v/v may be assayed to detect measurable Oxox activities.

3.3 INVESTIGATION OF THE SUBSTRATE SPECIFICITY OF SIGMA OXALATE OXIDASE

The substrate specificity of Oxox purified from numerous sources was discussed in chapter 1.6.4, as a result, the substrate specificity of Sigma Oxox was investigated to determine the reliability of the information in the literature. These studies were also performed to determine the likelihood of alternative substrates interfering when analysing biological samples.

The conventional substrate for Oxox is oxalate ($C_2H_2O_4$) which is a relatively small compound with a molecular mass of 90 Da comprising two directly linked carboxylic acid groups. Two sets of potential substrates of Oxox were selected, the first set of compounds contained linked di-acid groups with a similar structure to oxalate, the second set possessed a variety of functional groups. A conventional Oxox assay contains 1.31 mM oxalate, therefore, the substrates investigated were prepared to give this, and ten times the substrate concentration of 13.1 mM. Assays were performed using the MBTH assay with oxalate being substituted by each potential substrate being investigated.

3.3.1 Substrate Specificity using Structurally Related Possible Substrates

A number of compounds with similar structures to oxalate were selected, since it was thought these compounds would be more likely to be oxidised by Oxox. Both acid groups of the oxalate structure were retained allowing variation in the linking unit. The substrates selected were malonate ($CH_2(COOH)_2$): comprising an additional CH_2, phthalate ($C_8H_6O_4$): introducing an aromatic group, maleate and fumarate ($C_4H_4O_4$): both comprising an additional vinyl linkage with maleate being the cis form and fumarate the trans. The structure of oxalate and the selected compounds are shown in figure 3.2.

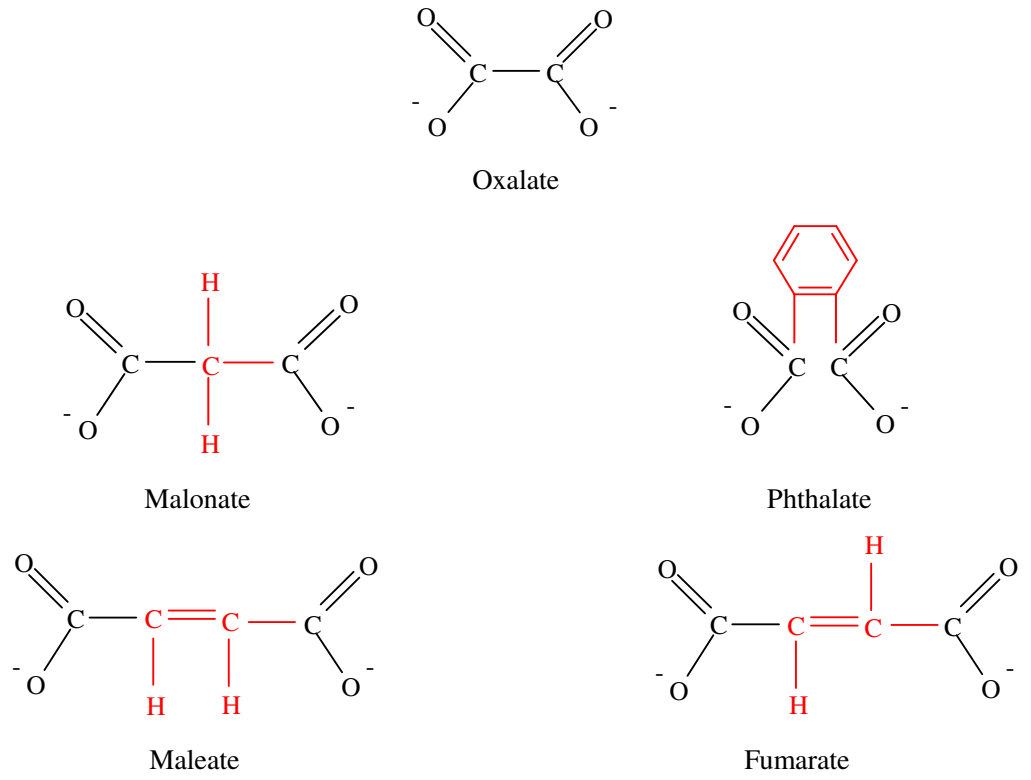


Figure 3.2: Structurally Related Possible Substrates for Sigma Oxalate Oxidase.
The structural changes in comparison to oxalate are highlighted in red and all substrates used were sodium based salts.

Two of the four compounds investigated during these studies: malonic acid [80, 84] and maleic acid [72, 80, 84] have been investigated as possible substrates for Oxox purified from barley seedling [84], *Pseudomonas* sp. OX-53 [80] and sorghum leaves [72]. Oxox purified from these sources were reported not to be oxidised by malonic and maleic acid. However, it is not reported in the literature whether the remaining two compounds, phthalic acid and fumaric acid have been investigated as potential substrates.

The compounds studied in figure 3.2 were found not to be substrates for Oxox at either the substrate concentrations used. These findings were comparable with those published by Koyama [80], Sugiura *et al*. [84], and Satyapal and Pundir [72] who reported Oxox to be specific for oxalate as a substrate.

3.3.2 Substrate Specificity using Possible Substrates with Different Functional Groups

Further potential substrates of Sigma Oxox were selected which possessed an additional functional group. The oxalate structure was used as a template and compounds with different functional groups ranging from a methyl to a vinyl group were selected. The compounds selected were pyruvate ($C_3H_3O_3$): comprising a methyl alcohol group, lactate ($C_3H_6O_3$): introducing an ethyl group, acrylate (C_3H_4O): comprising a vinyl group and glycolate ($HOCH_2COOH$): introducing an alcohol group. The structures of the potential substrates are shown in figure 3.3 alongside the structure of oxalate.

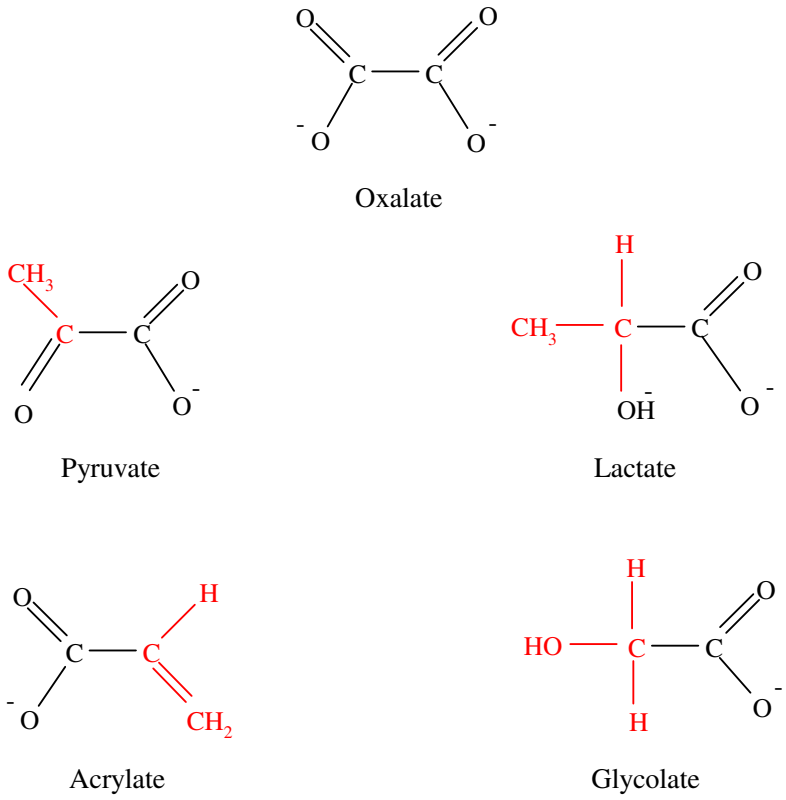


Figure 3.3: Possible Substrates for Sigma Oxalate Oxidase Comprising Different Functional Groups.

Possible substrates possessing different functional groups are highlighted in red and all compounds used were sodium based salts.

Pyruvic and glycolic acid are both oxalate precursors metabolised via the glycolylic pathway, therefore, the oxidation of these compounds was of great interest.

Glycolic acid appears to be the only compound from the possible substrates in figure 3.3 to

have been investigated as a potential substrate for purified Oxox. Glycolic acid has been found not to be oxidised by Oxox in barley seedlings [84] and *Pseudomonas* sp. OX-53 [80].

The potential substrates investigated during these studies did not generate hydrogen peroxide at either concentrations of 1.31 mM or 13.1 mM. The inability to detect hydrogen peroxide indicated these compounds were not substrates for commercially available Sigma Oxox. Sugiura *et al*. [84], and Koyama's [80] finding were consistent with those obtained during these studies since Sigma Oxox did not oxidise glycolic acid.

In conclusion, the substrate specificity studies undertaken demonstrated the absolute requirement for two directly connected carboxylic acid groups, suggesting Oxox activity is reliant upon the cleavage of the bond connecting the acid groups. The retention of both acid groups within the compounds investigated showed no Oxox activity, therefore, the linkage of the two acid groups is equally important in facilitating the enzyme reaction.

3.4 PURIFICATION OF SIGMA OXALATE OXIDASE

The aim of this research was to isolate and purify an alternative source of Oxox. As a stepping stone to this, a working purification protocol for commercially available Sigma Oxox was developed to standardise procedures.

Oxox has been purified from many sources by a variety of procedures such as subcellular fractionation, ammonium sulphate fractionation, thermal treatment, gel filtration chromatography, ion exchange chromatography, chromatofocussing, isoelectric focussing and affinity chromatography, discussed in chapter 1.5. Certain purification procedures are more favoured by research groups than others. A combination of these procedures were employed to purify the relatively impure Sigma Oxox. To initiate the purification of Sigma Oxox, cation exchange chromatography was undertaken.

3.4.1 Cation Exchange Chromatography

Cation exchange chromatography using carboxymethyl (CM) cellulose has been successfully used to purify recombinant Oxox expressed in *Pichia pastoris* [77]. CM cellulose cation exchanger (method 2.9.1.1) was used to achieve the desired purification of

Sigma Oxox, in accordance with the procedure performed by Requena and Bornemann to purify Oxox from barley roots [85].

Preparations of Sigma Oxox applied and eluted from the cation exchange column were assayed using both + and – oxalate MBTH assay mixtures. The resultant chromatogram following chromatography is shown in figure 3.4.

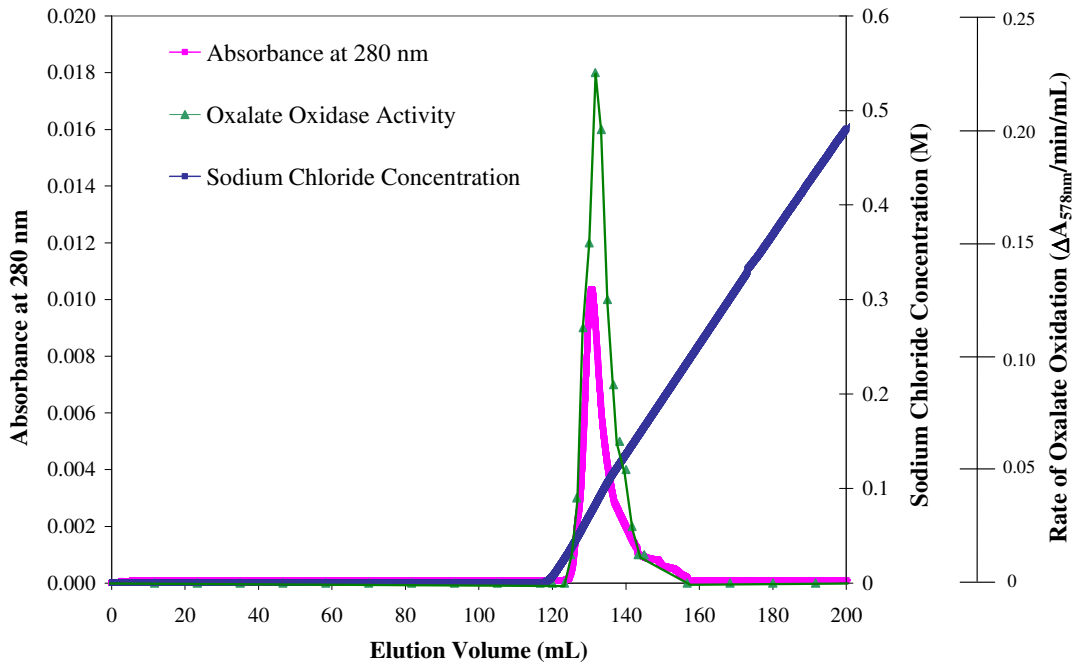


Figure 3.4: Cation Exchange Chromatography of Sigma Oxalate Oxidase.

3 U/5 mL Oxox prepared in 45 mM sodium succinate pH 3.8 was applied to the CM cellulose column and eluted using 0 to 1 M sodium chloride gradient. Cation exchange chromatography was performed following method 2.9.1.1, all eluted fractions were assayed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate.

Enzymatic assays performed after cation exchange chromatography detected Oxox activity within the fractions eluted with approximately 0.1 M sodium chloride, which is in accordance with Requena and Bornemann's data [85]. Although Oxox eluted at the expected point within the salt gradient, the percentage yield was low being 32.4 ± 3.9 %.

The poor yields suggested the column may be retaining the residual enzyme. To investigate this, the related enzyme glucose oxidase (Gox) was subjected to cation exchange chromatography under the same experimental conditions. The studies with Gox gave near 100 % yield (results not shown), as a result, the stability of Sigma Oxox became a target for investigation.

3.4.1.1 Stability of Sigma Oxalate Oxidase

The poor percentage yield of Sigma Oxox after cation exchange chromatography suggested the enzyme may be unstable at room temperature (approximately 22 °C) over 6 to 7 hours, the environment in which chromatographic analysis was undertaken. Therefore, the length of time and the dilution of the enzyme as it eluted during cation exchange chromatography may have led to its denaturation. To investigate this, Oxox was diluted 20, 100 and 200 fold in the buffer used during cation exchange chromatography: 10 mM sodium acetate pH 5.0. These enzyme preparations were assayed for Oxox activity at zero time and after 10 hours after being stored at 4 °C and approximately 22 °C beside the cation exchange column to mimic the environment the chromatography was performed. The results are shown in table 3.3.

	Oxalate Oxidase Activity (ΔA_{578nm}/min/mL)		
	Prior to Incubation	**Stored at 4 °C after 10 Hours**	**Stored at 22 °C after 10 Hours**
Neat	1.578 ± 0.015	1.590 ± 0.018	1.540 ± 0.011
1/20	0.087 ± 0.008	0.079 ± 0.010	0.080 ± 0.015
1/100	0.026 ± 0.015	0.027 ± 0.017	0.027 ± 0.002
1/200	0.013 ± 0.003	0.013 ± 0.004	0.014 ± 0.001

Table 3.3: The Stability of Sigma Oxalate Oxidase.

Neat Oxox (0.1 U/100 µL) was prepared in 10 mM sodium acetate pH 5.0. Assays were performed using 100 µL prior to and after incubation at 4 °C and approximately 22 °C for 10 hours using the MBTH assay method 2.12.6 at A_{578nm} in triplicate.

The Oxox activities measured after diluting and storing Oxox preparations at 4 °C and 22 °C were very similar. These studies indicated Sigma Oxox was stable at room temperature (approximately 22 °C) for 10 hours and diluting the enzyme up to 200 fold did not affect its activity.

3.4.1.2 Effect of Sodium Chloride on the Enzymatic Assay and Sigma Oxalate Oxidase

To further investigate the cause of the loss of Oxox activity during cation exchange chromatography, the effect of sodium chloride on the MBTH assay components and Sigma Oxox were determined. This was undertaken since sodium chloride was employed to elute bound Oxox from the cation exchange column.

3.4.1.2.1 The Effect of Sodium Chloride on the MBTH Enzymatic Assay

To ascertain whether sodium chloride had an effect on the dye precursors within the MBTH assay, the assay mixture was prepared containing a series of sodium chloride concentrations ranging from 0 to 2 M. Conventionally, the oxidation of oxalate by Oxox generates hydrogen peroxide, this in turn reacts with the dye precursors and horseradish peroxidase (HRP) to yield a coloured dye. Hence, Oxox was replaced by hydrogen peroxide in the assay mixture to mimic the end product of the oxidation of oxalate.

Assays were performed immediately after the addition of sodium chloride to the assay mixture containing the dye precursors and after storing the same mixture at 4 °C for 24 hours. The assays performed immediately, and after 24 hours were done so to investigate whether sodium chloride had an instant or prolonged affect on the MBTH assay components. The results obtained are shown in figure 3.5.

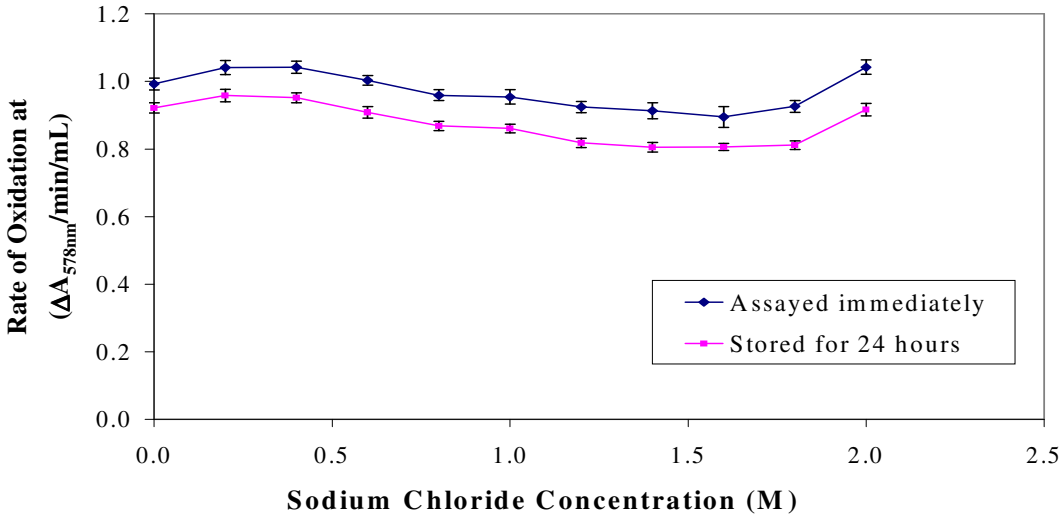


Figure 3.5: The Effect of Sodium Chloride on the MBTH Assay.

Assays were conducted immediately after the addition of sodium chloride to the assay mixtures and after storing preparations at 4 °C for 24 hours. Assays were performed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate. Error bars are a representation of standard deviation, N=3.

Less than 10 % reduction in activity was observed at any concentration of sodium chloride, whether assayed immediately or after 24 hours, suggesting this salt had a negligible effect on the MBTH assay.

3.4.1.2.2 The Effect of Sodium Chloride on Sigma Oxalate Oxidase

To determine if Sigma Oxox was stable in sodium chloride, the enzyme was prepared in a series of salt concentrations ranging from 0 to 2 M. Assays were performed immediately after the addition of sodium chloride and after storing Oxox preparations for 24 hours at 4 °C, the results are shown in figure 3.6.

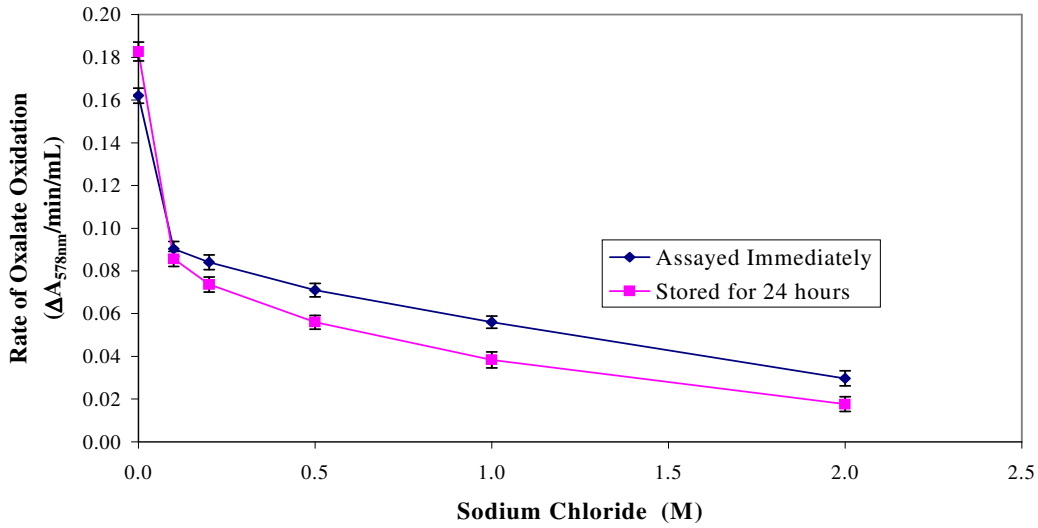


Figure 3.6: The Effect of Sodium Chloride on Sigma Oxalate Oxidase Activity.

Sigma Oxox was prepared in 45 mM sodium succinate pH 3.8, assays were performed immediately after the addition of sodium chloride and after storing preparations at 4 °C for 24 hours. All assays were performed using 0.1 U Oxox following the MBTH assay method 2.12.6 at A_{578nm} in triplicate. Error bars are a representation of standard deviation, N=3.

The effect of sodium chloride on Sigma Oxox is visible from figure 3.6 since Oxox activity decreased substantially. The addition of 0.1 M sodium chloride to Sigma Oxox had an instantaneous effect on Oxox and led to the reduction of approximately 55 % activity. Higher concentrations of sodium chloride, for example, 2 M had a detrimental immediate effect on Oxox activity reducing it by approximately 85 %.

3.4.1.2.3 Overview of the Effect of Sodium Chloride on the Enzymatic Assay and Sigma Oxalate Oxidase

Primarily, the effect of sodium chloride on the MBTH assay components was studied and it was found that this salt did not have an affect on the MBTH assay. These finding led to further investigation into the effect of sodium chloride on Sigma Oxox itself. Sigma Oxox activities were profoundly inhibited as increased sodium chloride concentrations were added to enzyme preparations.

These findings were in accordance with those published where 0.1 M sodium chloride inhibited Oxox activity in barley roots by 14 % [78] and barley seedlings by 57 % [84]. Lower concentrations of sodium chloride have also been reported to inhibit Oxox activity, for example 0.2 mM by 32 % in moss [124].

3.4.1.3 Investigating the Use of an Alternative Salt Elution Buffer for Cation Exchange Chromatography

To continue using cation exchange chromatography, an alternative salt to sodium chloride for the use with this procedure was investigated.

It is evident from the preceding subsection that sodium chloride inhibited Sigma Oxox activity, however, it was unclear whether chloride ions or sodium ions within the sodium chloride buffer had an effect on Oxox. To identify if the loss of activity was due to chloride ions, Sigma Oxox was prepared in a series of potassium chloride and magnesium chloride concentrations ranging from 0 to 1 M. Enzyme preparations were assayed immediately after the addition of each salt and after storing at 4 °C for 24 hours. The results obtained are shown in figure 3.7a (potassium chloride) and figure 3.7b (magnesium chloride).

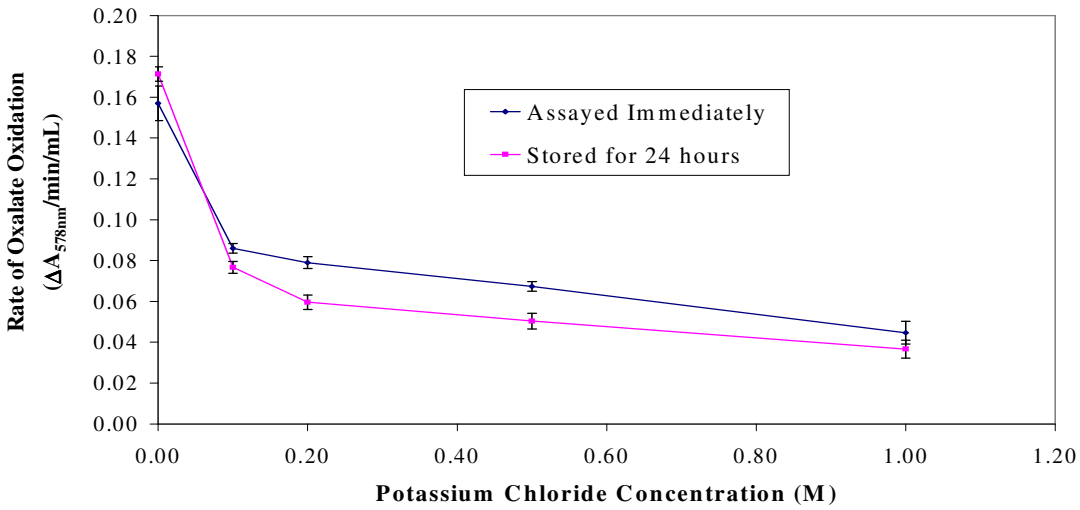


Figure 3.7a: The Effect of Potassium Chloride on Sigma Oxalate Oxidase Activity.
Sigma Oxox was prepared in 45 mM sodium succinate pH 3.8, assays were performed in triplicate with 0.1 U Oxox using the MBTH assay method 2.12.6 at A_{578nm} immediately after the addition of the salt and after storing at 4 °C for 24 hours. Error bars are a representation of standard deviation, N=3.

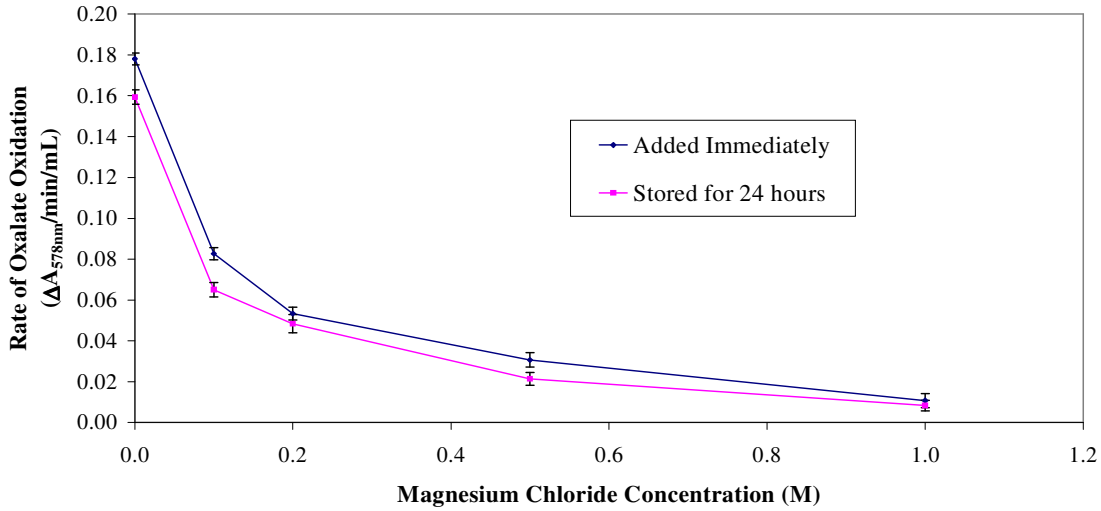


Figure 3.7b: The Effect of Magnesium Chloride on Sigma Oxalate Oxidase Activity. Sigma Oxox was prepared in 45 mM sodium succinate pH 3.8, assays were performed in triplicate with 0.1 U Oxox using the MBTH assay method 2.12.6 at A_{578nm} immediately after the addition of the salt and after storing at 4 °C for 24 hours. Error bars are a representation of standard deviation, N=3.

The immediate addition of 0.1 M potassium chloride to enzyme preparations led to the reduction of 45 % Oxox activity, similar results were obtained after the immediate addition of 0.1 M magnesium chloride, where Oxox activity reduced by approximately 55 %. The instability of Sigma Oxox in potassium chloride and magnesium chloride suggested of chloride ions had a detrimental effect on Sigma Oxox.

Further stability studies with sodium sulphate were undertaken to establish whether Oxox was stable in the presence of sodium ions since it was evident chloride ions reduced Oxox activity. As with previous studies, assays were conducted immediately after the addition of sodium sulphate (0 to 0.5 M) and after being stored at 4 °C for 24 hours, the results obtained are shown in figure 3.8.

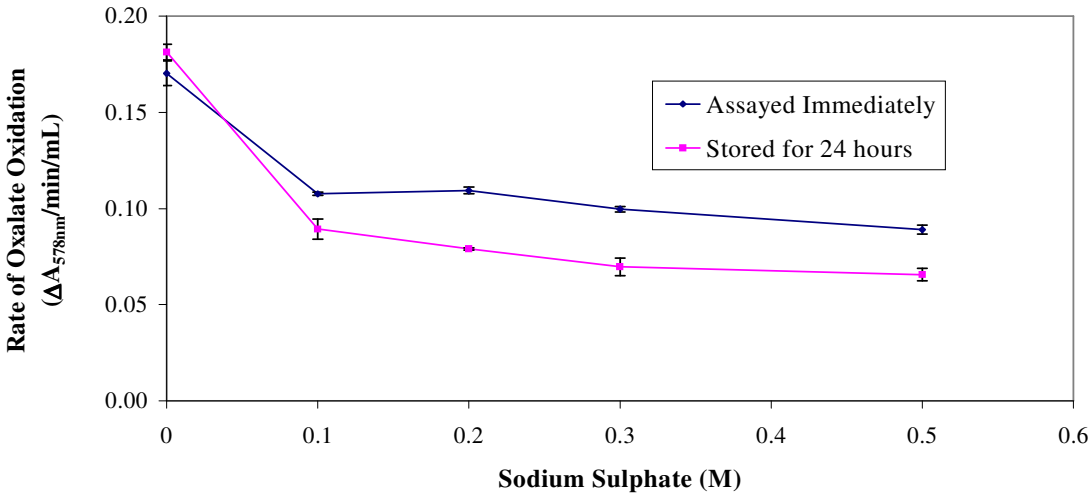


Figure 3.8: The Effect of Sodium Sulphate on Sigma Oxalate Oxidase Activity.

Sigma Oxox was prepared in 45 mM sodium succinate pH 3.8, enzymatic assays were performed in triplicate immediately after the addition of sodium sulphate and after storing at 4 °C for 24 hours. Assays were undertaken using 0.1 U Oxox following the MBTH assay method 2.12.6 at A_{578nm}. Error bars are a representation of standard deviation, N=3.

Preparing Sigma Oxox with sodium sulphate did not reduce its activity as adversely as sodium chloride, potassium chloride or magnesium chloride. Nevertheless, the addition of 0.1 M sodium sulphate led to 36 % loss of Oxox activity.

It was clear from these studies that Sigma Oxox was not stable in the salt solutions containing either chloride ions or sodium ions. Considering this, the stability of Oxox in manganese chloride was investigated since barley root Oxox has been reported to contain manganese as a cofactor and plays an important role in its activity [85].

Oxox was prepared in series of manganese chloride concentrations ranging from 0 to 1 M, enzymatic assays were performed immediately after the addition of this salt and after storing enzyme preparations at 4 °C for 24 hours, the results are shown in figure 3.9.

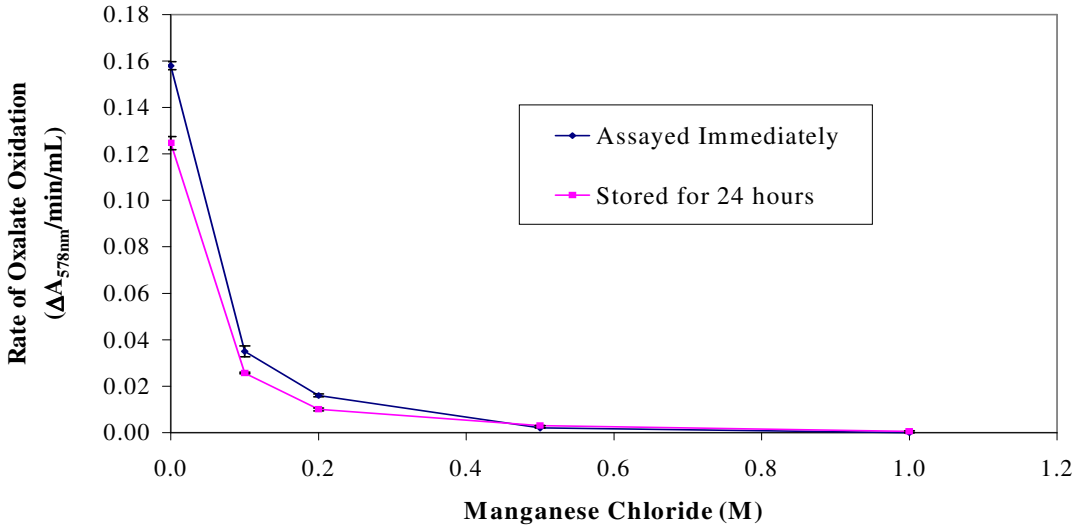


Figure 3.9: The Effect of Manganese Chloride on Sigma Oxalate Oxidase Activity.

Sigma Oxox was prepared in 45 mM sodium succinate pH 3.8, enzymatic assays were performed immediately after the addition of manganese chloride and after storing at 4 °C for 24 hours. Assays were performed using 0.1 U Oxox with the MBTH assay method 2.12.6 at A_{578nm} in triplicate. Error bars are a representation of standard deviation, N=3.

It is not clear from the literature whether the addition of manganese chloride to Sigma Oxox would stimulate or inhibit the activity. Manganese has been reported to inhibit Oxox activity in barley seedlings by 3 % (1 mM) [84] and 44 % (7.5 mM) [87], conversely, manganese ions stimulated Oxox activity in *Pseudomonas* sp. OX-53 by 75 % (0.1 mM) [80].

The addition of 0.1 M manganese chloride inhibited Sigma Oxox activity by 78 %. Manganese ions appeared to have a greater detrimental effect on Sigma Oxox in comparison to the previous salts investigated, since the enzyme activity decreased more profoundly.

In conclusion, these studies led to the finding that Sigma Oxox was not stable in any of the salt solutions investigated suggesting the overall ionic strength of the solution may be the contributing factor rather than a particular salt or ionic species.

3.4.2 Affinity Chromatography of Sigma Oxalate Oxidase

The purification of Sigma Oxox was pursued further by affinity chromatography following Requena and Bornemann's purification protocol [85] using Concanavalin A-Sepharose 4B

affinity media. Two buffers are employed in this procedure: 2 mM methyl-α-D-mannopyroside and 50 mM sodium acetate pH 5.0 containing 0.1 M sodium chloride, 1 mM calcium chloride and 1 mM magnesium chloride. Since Sigma Oxox activity was inhibited by sodium chloride and magnesium chloride, the acetate buffer was prepared in the absence of these salts.

Prior to the purification of Oxox using affinity chromatography following method 2.10, the stability of Sigma Oxox in the buffers used during this procedure (2 mM methyl-α-D-mannopyroside and 50 mM sodium acetate pH 5.0) was investigated and the enzyme was found to be stable. Oxox preparations applied to the affinity column were assayed in addition to the eluted fractions to determine the percentage yield. The resultant chromatograph is shown in figure 3.10.

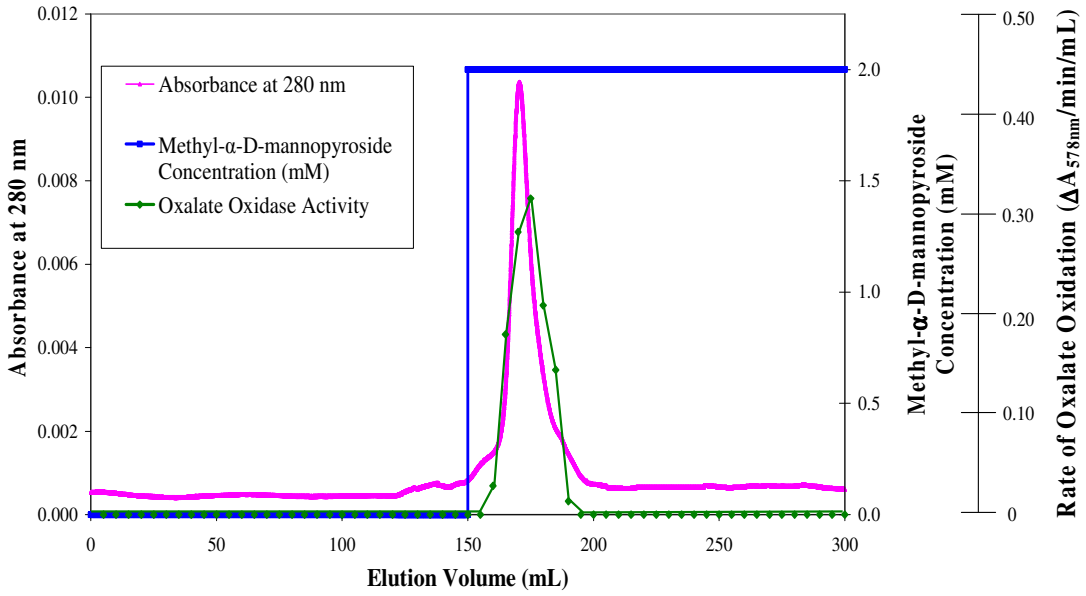


Figure 3.10: Affinity Chromatography of Sigma Oxalate Oxidase.

3.0 U of Oxox was prepared in 5 mL 45 mM sodium succinate pH 3.8 and eluted using 50 mM sodium acetate pH 5.0, and 2 mM methyl-α-D-mannopyroside (prepared in 50 mM sodium acetate pH 5.0) following method 2.10. All fractions eluted were assayed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate.

Oxox eluted from the affinity column after the introduction of methyl-α-D-mannopyroside. Although this procedure led to the successful elution of Oxox, the yield of Oxox activity eluted was very low being 67.3 ± 5.9 %.

Considering Sigma Oxox was found to be stable in the buffers used during this procedure,

the loss of activity was probably caused by an additional factor: Oxox may be retained within the chromatographic system.

Affinity chromatography was performed using the AKTA prime® purification system which contains an inbuilt filtering mechanism. All buffers used were filtered prior to use, however, preparations containing Sigma Oxox were purified without being filtered due to the small volumes used i.e. only 5 mL. Therefore, the loss of activity may be due to the incomplete dissolution of Sigma Oxox which would have led to the removal of the undissolved enzyme by the filtration mechanism within the ÄKTA prime® system. To confirm this, investigations regarding the solubility of Oxox were undertaken.

3.4.2.1 Solubility of Sigma Oxalate Oxidase

Since the solubility of Sigma Oxox had now become a target for investigation, series of experiments were conducted to determine the solubility of Sigma Oxox. Oxox (0.1 U/100 µL) was prepared in 45 mM sodium succinate pH 3.8, after which, samples were centrifuged at relatively low speeds of 15,000 x g to remove undissolved enzyme. All enzyme preparations were assayed to measure Oxox activity prior to and after centrifugation.

The sample assayed prior to centrifugation possessed Oxox activity, however, the supernatant collected after centrifugation did not. The resultant pellet component after centrifugation was redissolved in the same buffer and assayed. Surprisingly, the pellet component possessed Oxox activity, as a result, it was concluded Sigma Oxox was not dissolving fully. To aid the dissolution of Oxox, samples were stirred for 24 and 48 hours at 4 °C followed by centrifugation. The supernatant collected after centrifugation at 15,000 x g was assayed and did not possess Oxox activity.

To investigate the dissolution of Oxox further, neat, 1/2, 1/5, 1/10, 1/20 and 1/40 preparations of Oxox were vortex mixed, followed by centrifugation to establish if Oxox would dissolve at lower concentrations. All samples were assayed after being mixed thoroughly using a vortex mixer and after centrifugation. The results obtained are shown in figure 3.11.

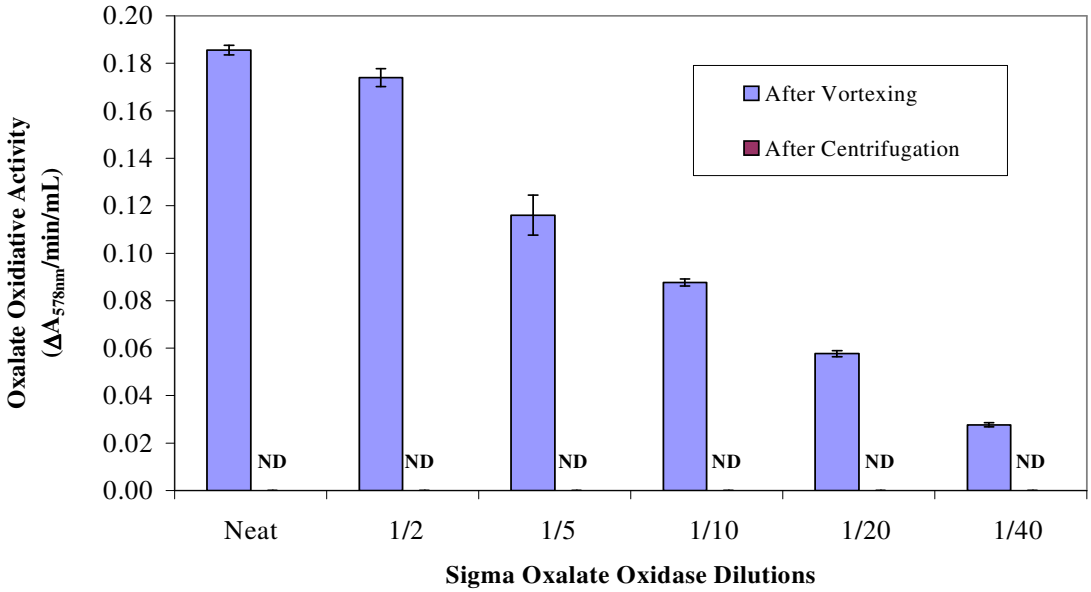


Figure 3.11: The Solubility of Sigma Oxalate Oxidase.

Neat Sigma Oxox (0.1 U/100 µL) preparations were assayed using 100 µL, subsequent dilutions of 1/2, 1/5, 1/10, 1/20 and 1/40 were assayed using 100 µL. Enzymatic assays were performed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate. Error bars are a representation of standard deviation. ND denotes none detected, N=3.

Vortex mixing Oxox preparations did not aid the dissolution of the enzyme, as shown in figure 3.11. All Oxox activity was found in the pellet component after centrifugation. To investigate the dissolution further, prepared samples were also sonicated, followed by centrifugation. Similar results were obtained, whereby Oxox activity was not detected in the supernatant component after centrifugation.

Following this, enzyme preparations were dialysed for 24 hours at 4 °C to aid its dissolution, after which the samples were centrifuged. Oxox preparations were assayed on three occasions, firstly, prior to dialysis, secondly, after dialysis and thirdly, after centrifugation. Oxox activity was detected in the samples assayed prior to and after dialysis, however, complete loss of Oxox activity was observed in the supernatant component after centrifugation.

The solubility studies demonstrated that diluting, vortex mixing, sonicating and dialysing Sigma Oxox preparations did not aid its dissolution in its optimum buffer (45 mM sodium succinate pH 3.8).

The insolubility of Sigma Oxox was therefore probably the contributing factor which led to

poor yields during affinity chromatography. Since inappropriate yields were also obtained during cation exchange chromatography, the salt sensitivity of Sigma Oxox coupled with its insolubility were likely to have caused this.

Having concluded Sigma Oxox was poorly soluble and unstable in certain salt solutions, further purification of this commercial enzyme was not pursued, however, amperometric analysis of this enzyme source was undertaken.

3.5 AMPEROMETRIC ANALYSIS

Initial electrochemical studies were performed using Sigma Gox as a model system rather than Sigma Oxox, due to the high cost of the latter. Initial experiments studied the effect of varying Gox concentrations on the platinum electrode surface to establish if the concentration of the enzyme had an effect on the signal produced by the biosensor.

Further amperometric analysis was undertaken to determine the repeatability and reproducibility of the Sigma Oxox sensor since it will be employed to detect oxalate in patients suffering from primary hyperoxaluria.

3.5.1 Amperometric Analysis of Sigma Glucose Oxidase

Varying concentration of Gox were immobilised onto a platinum electrode to determine if the concentration of the immobilised enzyme would affect the electrode sensor response. This information will be useful in determining the concentration of immobilised Oxox required to obtain an adequate sensor response to detect oxalate, since the concentration of purified Oxox from alternative sources may differ.

Amperometric studies were recorded by measuring the current response produced by hydrogen peroxide verses time. Sequential additions of glucose (equivalent to 1 mM) were added during Gox amperometric studies. An increase in the current response was observed after each addition of glucose, an example of such a plot is shown in figure 3.12.

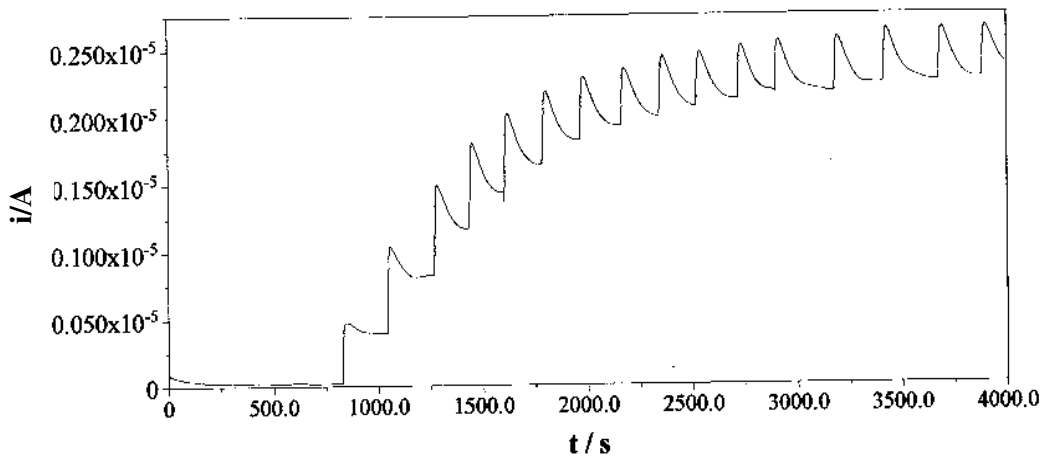


Figure 3.12: An Example of a Current Versus Time Plot During Amperometric Analysis of Glucose Oxidase.

To determine the effect of altering the concentration of Gox at the electrode surface, varying concentrations of this enzyme were prepared in the STY1 polymer to give final concentrations of 4.0, 6.0 and 8.0 U/µL. Amperometric analysis was conducted following method 2.18. The data was extracted from each series of experiment and is shown in figure 3.13.

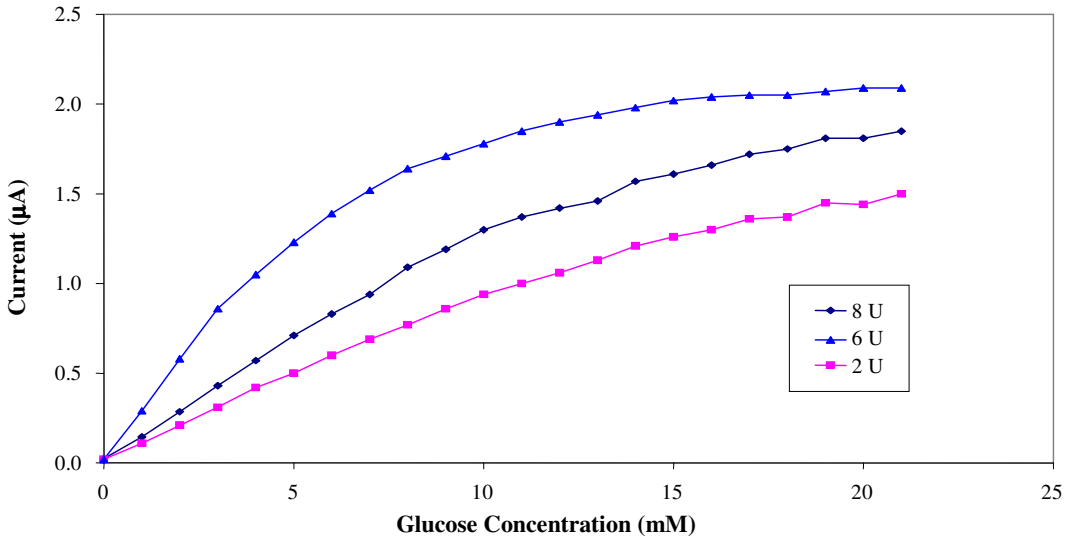


Figure 3.13: Comparison of Varying Concentrations of Glucose Oxidase Immobilised on a Platinum Biosensor.

Sequential additions of 1 mM glucose were added during each amperometric analysis following method 2.18. Enzyme concentrations of 4, 6 and 8 U were prepared per µL.

The highest current response observed during these studies was with the immobilisation of 6 U/µL Gox on to the platinum electrode. When more than 6 U/µL Gox was immobilised,

a decrease in the current response was observed. This may be due to a combination of two factors, firstly, the maximum enzyme concentration which could be contained in the membrane was 6 U, therefore, the excess enzyme would have been excluded during drying. Secondly, as the enzyme loading increased, the membrane thickness also increased which led to the overall decrease in the current response since this represents an increased diffusion barrier to both hydrogen peroxide and glucose.

3.5.2 Amperometric Analysis of Sigma Oxalate Oxidase

Once preliminary amperometric analysis was performed using Gox, the repeatability and reproducibility of a Sigma Oxox biosensor was investigated to determine the shelf life and operational stability. This was undertaken since these factors will have implications for the Oxox biosensor once it is fabricated using purified Oxox. The reproducibility and repeatability of the Oxox biosensor is also required to be of a high standard to generate accurate and reliable results.

Oxox was prepared in the STY1 polymer in a similar manner to previous studies and was repeated on three independent occasions to determine the reproducibility of such a biosensor system. Figure 3.14 shows the reproducibility of the Oxox biosensor.

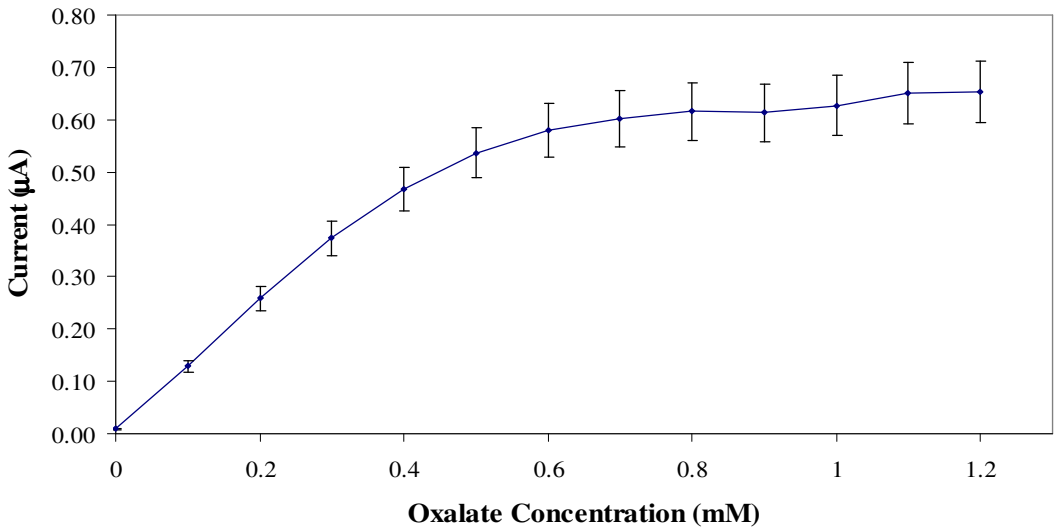


Figure 3.14: The Reproducibility of an Oxalate Oxidase Biosensor.

Amperometric analysis was performed on three independent occasions in triplicate following method 2.18. Sequential additions of 0.1 mM oxalate were added. Error bars are a representation of standard deviation, N=3.

The preparation of the Oxox sensor on three independent occasions led to very similar

results, illustrating the successful reproducibility of the oxalate sensor. The linear range of the sensor was from 0 to 0.5 mM, which is within the detectable oxalate limit of 450 µM.

The linear range of this Sigma Oxox biosensor was compared with the linear range of other developed biosensors. The linear ranges of the biosensors developed by Milardović *et al*. of 2.5 µM to 400 µM [31], 0 to 100 µM [140], and Reddy *et al*. of 0 to 60 µM [35] oxalate are lower than the linear range of the Sigma Oxox sensor developed.

Amperometric analysis was also undertaken to determine the repeatability of the Oxox biosensor. Multiple amperometric analyses were carried out using the same platinum electrode which had been soaked for two hours prior to conducting further repeatability studies. These experiments were performed to ensure similar current responses were generated after the reuse of the same sensor. This will have implications when detecting oxalate in numerous urine samples avoiding the need for sensor replacement. The results obtained for such repeatability analysis is shown in figure 3.15.

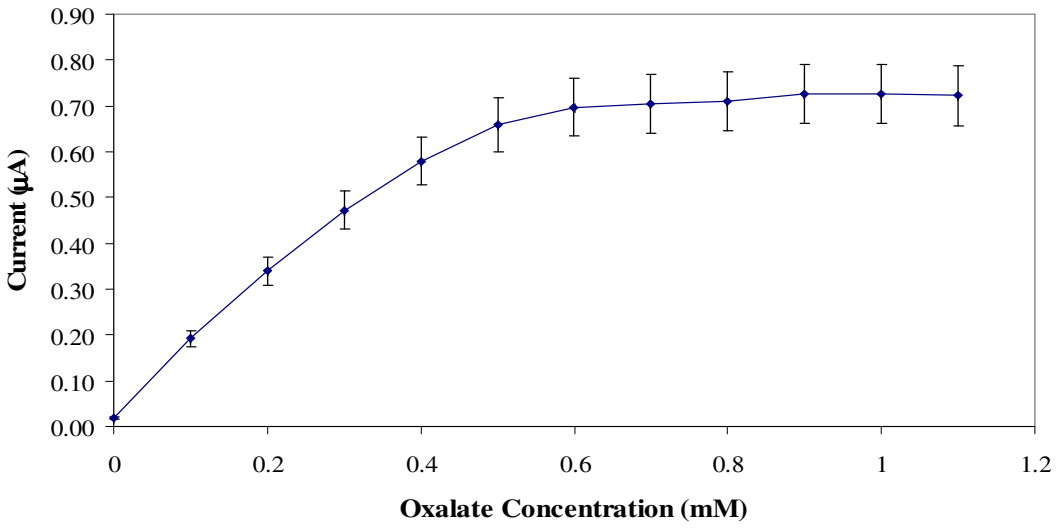


Figure 3.15: The Repeatability of an Oxalate Oxidase Biosensor.

Amperometric analysis (N=3) was performed in triplicate with the same biosensor which had been washed between experiments following method 2.18. Sequential additions of 0.1 mM oxalate were added. Error bars are a representation of the standard deviation.

The current response measured after repeating oxalate measurements using the same Oxox sensor were very repeatable confirming the ability to analyse numerous urine samples with the same electrode.

Since Sigma Oxox was successfully immobilised onto a platinum electrode, the established amperometric methodology will be further employed to immobilise Oxox purified from alternative sources.

3.6 CONCLUSION

Prior to the purification of Sigma Oxox, an optimum assay, substrate specificity, optimum buffer and pH were investigated. Enzymatic assays performed using the MBTH assay led to the detection of elevated Oxox activity and reduced standard error in comparison to the ABTS and aminophenazone assays. As a result, the MBTH assay was selected to be used during the purification of Sigma Oxox.

Two sets of potential substrates for Sigma Oxox were investigated, the first set possessing di-acid groups similar to the conventional substrate oxalate, and the second set containing a different functional group. Neither set of potential substrates were oxidised by Oxox, demonstrating the specificity of the commercial enzyme.

Optimum buffer conditions for Sigma Oxox were explored using 45 mM sodium succinate pH 3.8, 50 mM sodium phosphate pH 7.0 and 10 mM sodium acetate pH 5.0. Elevated Oxox activity was measured after preparing Oxox and assay mixtures in 45 mM sodium succinate pH 3.8 compared to those generated after preparing in the latter two buffers. The optimum pH of 45 mM sodium succinate was investigated and led to the finding that pH 3.8 was in fact the of optimum pH 45 mM sodium succinate.

The buffering capability of this buffer was examined to ensure the pH would not greatly alter after the addition of up to 20 % v/v 50 mM sodium phosphate pH 7.0 or 10 mM sodium acetate pH 5.0. Insignificant changes in the pH of 45 mM sodium succinate were observed after the addition of up to 20 % v/v of the specified buffers. These findings enable increased volumes of fractions to be assayed to detect low activities during the purification of an alternative source of Oxox.

The purification of Sigma Oxox was undertaken to establish standardised and working purification protocols which would be employed to purify Oxox from alternative sources. The purification of Sigma Oxox using cation exchange chromatography was successful, however, the total Oxox activity eluted from the column did not correspond to the total

activity applied, posing questions regarding its stability.

The stability of the commercial enzyme was studied and found to be stable at 4 °C and 22 °C for 10 hours. The stability of Oxox in sodium chloride was also investigated, these studies led to the reduction of Oxox activity by approximately 50 % after the immediate addition of 0.1 M sodium chloride. Similar results were generated with potassium chloride, magnesium chloride, sodium sulphate and manganese chloride, demonstrating the instability of Oxox. As a result, the purification of Sigma Oxox using cation exchange chromatography was not continued.

Sigma Oxox was successfully purified using affinity chromatography, however, the total Oxox activity applied to the column did not correspond to that eluted. These findings led to the suggestion that Sigma Oxox was not fully dissolving. A number of methods were attempted to aid the dissolution of Sigma Oxox, however, they were ineffective.

In conclusion, the purification of Sigma Oxox was more challenging than expected since the enzyme was found to be unstable in salt buffers and less soluble than usual enzyme preparations. As a result, further attempts to purify this enzyme were not pursued.

Initial amperometric analysis regarding the repeatability and reproducibility of the Sigma Oxox biosensor was very successful demonstrating the reusability and reproducibility of the Oxox sensor. The established amperometric protocol will be employed to immobilise purified Oxox from alternative sources to develop a novel Oxox.

CHAPTER 4

PURIFICATION OF BARLEY ROOT OXALATE OXIDASE

The attempted purification of Sigma oxalate oxidase (Oxox), which was discussed in the preceding chapter was not as successful as expected due to dissolution problems, loss of Oxox activity during chromatographic procedures and the instability of the enzyme in salt buffers. Since the commercial enzyme is purified from barley roots, the purification of Oxox from this source was the first target for investigation.

Barley is a member of the *Poaceae* family, the leaves [67], roots [44, 76, 78, 85, 86] and seedlings [84, 97, 122] have been reported to contain Oxox. The purification of Oxox from barley roots is well documented and employs a combination of purification procedures, however, the protocols differ greatly reflecting the age of the literature and the contrasting characteristics and properties of the purified enzyme, discussed in chapter 1.6.

The purification of barley root Oxox was initiated by homogenisation followed by subcellular fractionation. Subsequently, a combination of procedures were employed including thermal treatment, ammonium sulphate fractionation, cation exchange chromatography and gel filtration chromatography. The procedures used were optimised to provide a relatively pure form of the enzyme of interest, further aiding the purification and isolation of additional sources of Oxox.

4.1 ISOLATION AND OPTIMUM ENZYMATIC ASSAY OF BARLEY ROOT OXALATE OXIDASE

To purify Oxox from barley roots, the crop was cultivated following method 2.4.1, once cultivated, Oxox from barley roots was isolated using subcellular fractionation according to method 2.5.1. The location of Oxox purified from different sources varies, with some being soluble [24, 28, 44, 67, 69, 70, 72, 75, 78, 82-85, 87, 90, 95, 97, 104, 109, 122, 124, 126] and others being insoluble, membrane bound [26, 73, 74, 79, 98, 103, 121]. Considering this, both the soluble (supernatant) and insoluble components (pellet) were investigated. The insoluble, membrane bound components were treated with sodium deoxycholate to release bound components following method 2.5. The insoluble components were assayed prior to and after treatment, in addition to the soluble components to detect the presence of Oxox.

To determine an optimum enzymatic assay, Oxox activity was measured using the ABTS, aminophenazone and MBTH assays, similar to the studies performed in chapter 3.1. The most sensitive assay selected during these studies would be employed throughout the purification of Oxox from barley roots ensuring the detection of low Oxox activities.

Assays were performed using two types of assay mixtures, the first containing the conventional substrate oxalate (+) and the second prepared in the absence of oxalate (–). The activities observed after assaying using both types of mixtures were used to calculate the 'corrected activity'. The activities measured in both the soluble and released membrane components of barley roots using the ABTS, aminophenazone and MBTH assays are illustrated in table 4.1.

	Oxalate Oxidase Activity (ΔA/min/mL)		
	+ Oxalate	**- Oxalate**	**Corrected Activity**
ABTS	**0.412 ± 0.002**	**0.064 ± 0.001**	**0.348 ± 0.002**
ABTS	**ND**	**ND**	**ND**
Aminophenazone	**0.395 ± 0.016**	**0.085 ± 0.014**	**0.310 ± 0.021**
Aminophenazone	**ND**	**ND**	**ND**
MBTH	**0.489 ± 0.008**	**0.078± 0.005**	**0.411 ± 0.024**
MBTH	**ND**	**ND**	**ND**

Table 4.1: Oxalate Oxidase Activity in Barley Root.

Oxox activity of the **soluble components** and **released membrane components after sodium deoxycholate treatment** were monitored at A_{650nm}, A_{520nm}, and A_{578nm}, for the ABTS, aminophenazone and MBTH assays respectively, following method 2.12.6 in triplicate. Activities below 0.010 ΔA/min/mL are labelled ND denoting 'none detected', N=3.

The enzymatic assays performed did not lead to the detection of Oxox activity within the released membrane components of barley roots either prior to (results not shown) or after sodium deoxycholate treatment. In contrast, the soluble component of barley roots contained activity after being analysed by both types of assay mixtures. The Oxox activity measured using – oxalate assay mixture was not expected. It was proposed this 'background' activity may be due to the presence of interfering species in the crude barley root extract interacting with the dye precursor and/or HRP causing a change in intensity of the dye within the assay mixture.

The activity observed using the – oxalate assay mixture was not classified as 'oxalate oxidase activity' since oxalate was not present in the assay mixture. As a result, the activity generated using the – oxalate assay mixture was termed 'oxidase activity'.

Assays performed using the MBTH assay produced the highest 'corrected activity' in comparison to the ABTS and aminophenazone assays. Taking this into account, the MBTH assay was selected for use with barley roots since it would detect relatively low Oxox concentrations during the purification of Oxox from this source.

The successful extraction of Oxox from barley roots using subcellular fractionation led to its further purification by thermal treatment.

4.2 THERMAL TREATMENT OF BARLEY ROOT OXALATE OXIDASE

The purification of barley root Oxox was pursued by thermal treatment which eliminated thermally unstable components. Extracts were assayed prior to and after thermal treatment at 60 ˚C for 10 minutes following method 2.6 using both + and – oxalate assay mixtures, the results obtained are shown in table 4.2.

	Oxalate Oxidase Activity (ΔA_{578nm}/min/mL)		
	+ Oxalate	**- Oxalate**	**Corrected Activity**
Prior to Thermal Treatment	0.426 ± 0.001	0.078 ± 0.001	**0.348 ± 0.001**
After Thermal Treatment	0.364 ± 0.001	0.048 ± 0.001	**0.316 ± 0.001**

Table 4.2: Thermal Treatment of Barley Roots.

Barley roots were thermally treated at 60 °C for 10 minutes following method 2.6. Assays were conducted using the MBTH assay method 2.12.6 at A_{578nm} in triplicate, N=3.

The Oxox activities measured using + and – oxalate assay mixtures after thermal treatment were reduced by approximately 15 % and 38 % respectively, demonstrating the successful purification of barley root Oxox. To concentrate the thermally treated barley root extract, ammonium sulphate fractionation (ASF) was performed.

4.3 AMMONIUM SULPHATE FRACTIONATION

Varying percentages of ammonium sulphate saturation have been reported to purify Oxox, most of which employ final saturations up to 80 % [24, 26-28, 67, 70, 72-74, 76, 80, 82-84, 120, 126]. Barley root Oxox was subjected to 30 % to 65 % ASF, following Pundir's [82], and Pundir and Nath's [83] protocols.

Enzymatic assays were performed prior to and after ASF (concentrated dialysate), the resultant activities measured after assaying using both + and – oxalate assay mixtures are shown in table 4.3.

	Oxalate Oxidative Activity (ΔA_{578nm}/min/mL)		
	+ Oxalate	**- Oxalate**	**Corrected Activity**
Prior to ASF	0.464 ± 0.001	0.088 ± 0.005	**0.376 ± 0.002**
After ASF	2.540 ± 0.002	0.011 ± 0.002	**2.530 ± 0.003**

Table 4.3: Ammonium Sulphate Fractionation of Thermally Treated Barley Roots.
Thermally treated barley root extract was subjected to 30 % to 65 % ASF following method 2.7 and dialysed against 10 mM sodium acetate pH 5.0 using method 2.8. Enzymatic assays were performed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate, N=3.

The Oxox activity measured after undertaking ASF substantially increased by approximately six fold. A reduction in the oxidase activity was also observed after performing ASF using the – oxalate assay mixture. This decrease was likely to be due to the removal of possible interfering species present in the extract prior to ASF.

Since the Oxox activities measured after concentrating the thermally treated barley root extract by ASF were over six fold greater than the crude extract, cation exchange chromatography was a viable purification option and was performed to further purify the enzyme preparation.

4.4 CATION EXCHANGE CHROMATOGRAPHY

To purify the extract further, cation exchange chromatography was employed using carboxymethyl (CM) cellulose cation exchange media. Prior to the purification of barley root Oxox using this procedure, the stability of the barley root extract in sodium chloride

was investigated. This was undertaken to ensure this procedure could be performed since Sigma Oxox was found to be unstable in salt buffers used during this procedure, discussed in chapters 3.4.1.2.2 and 3.4.1.3.

The stability studies performed led to the finding that the activity of barley root Oxox remained unchanged after the immediate addition of up to 2 M sodium chloride at 4 ˚C (results not shown). Having identified the stability of barley root Oxox in this salt, its purification by cation exchange chromatography was initiated following method 2.9.1.1.

The ASF concentrated barley root extract was assayed prior to its purification using cation exchange chromatography, additionally, all fractions eluted were assayed using both + and – oxalate mixtures. Conductivity and protein content were monitored online using a conductivity sensor and UV spectrophotometer indicating the concentration of salt required to elute the components and enabling the visualisation of eluted proteins respectively.

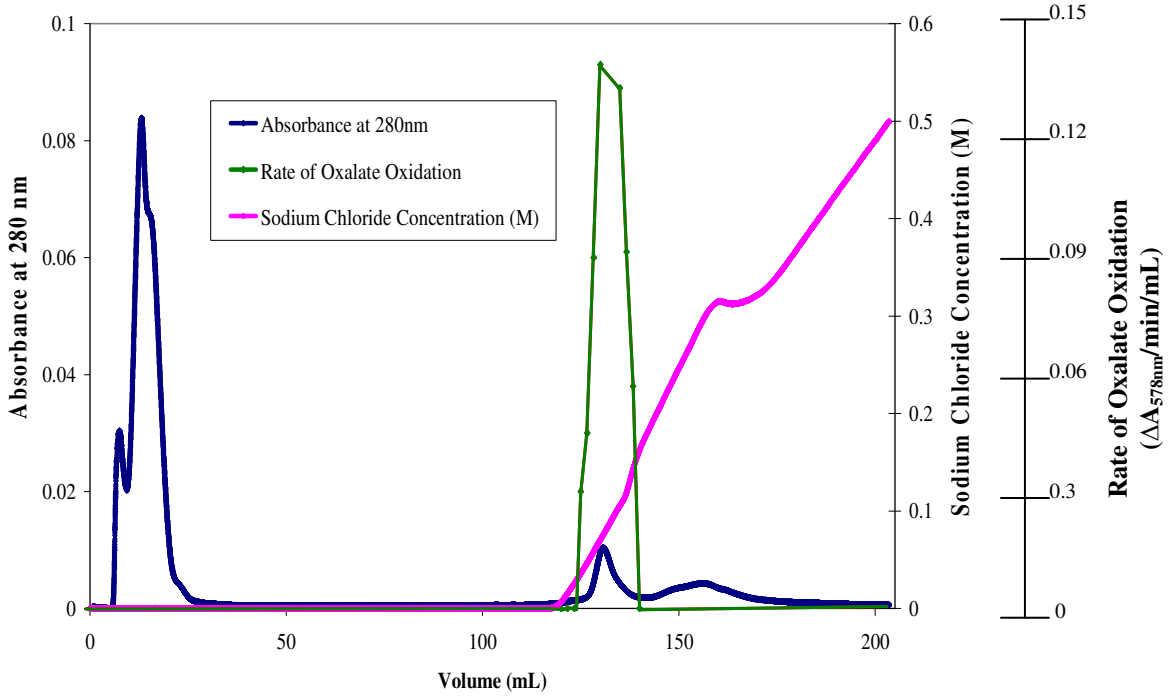


Figure 4.1: Cation Exchange Chromatography of Barley Root Oxalate Oxidase.

ASF Concentrated barley root Oxox was purified using CM cellulose cation exchange media and eluted with a 0 to 0.5 M sodium chloride gradient following method 2.9.1.1. Protein content at A_{280nm} and conductivity were monitored online. All fractions eluted (5 mL) were assayed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate.

The resultant chromatograph obtained after the purification of the ASF concentrated barley root Oxox using cation exchange chromatography is shown in figure 4.1.

The purification of barley root Oxox using cation exchange chromatography led to the elution of numerous protein peaks prior to and after the introduction of the sodium chloride gradient.

The components eluting prior to the sodium chloride gradient did not possess Oxox activity. Two distinctive peaks eluted during the sodium chloride gradient, at approximately 0.1 M and 0.3 M. The fractions assayed identified Oxox activity within the first peak eluting at approximately 0.1 M sodium chloride, similar to the findings obtained by Requena and Bornemann [85]. The purification of the ASF concentrated barley root Oxox resulted in the elution of approximately 87 % of the Oxox activity applied to the column, this is in contrast to the 23.9 % yield obtained with the commercial Sigma preparation in chapter 3.4.1.

To continue with the purification of Oxox from barley roots, the Oxox active fractions eluted from the cation exchange column were pooled and further purified using gel filtration chromatography.

4.5 GEL FILTRATION CHROMATOGRAPHY

Barley root Oxox was purified using Sephacryl S-200-HR gel filtration media, this media was also used to purify recombinant Oxox by Whittaker and Whittaker [77]. Two types of barley root extracts were purified using Sephacryl S-200-HR, firstly, the pooled Oxox active fractions after cation exchange chromatography and secondly, the concentrated extract after ASF (prior to its purification using cation exchange chromatography). The purification of both extracts using gel filtration chromatography was performed following method 2.11.2.

The resultant chromatograms obtained after gel filtration chromatography are shown in figure 4.2a (after ASF) and figure 4.2b (after ASF and cation exchange chromatography).

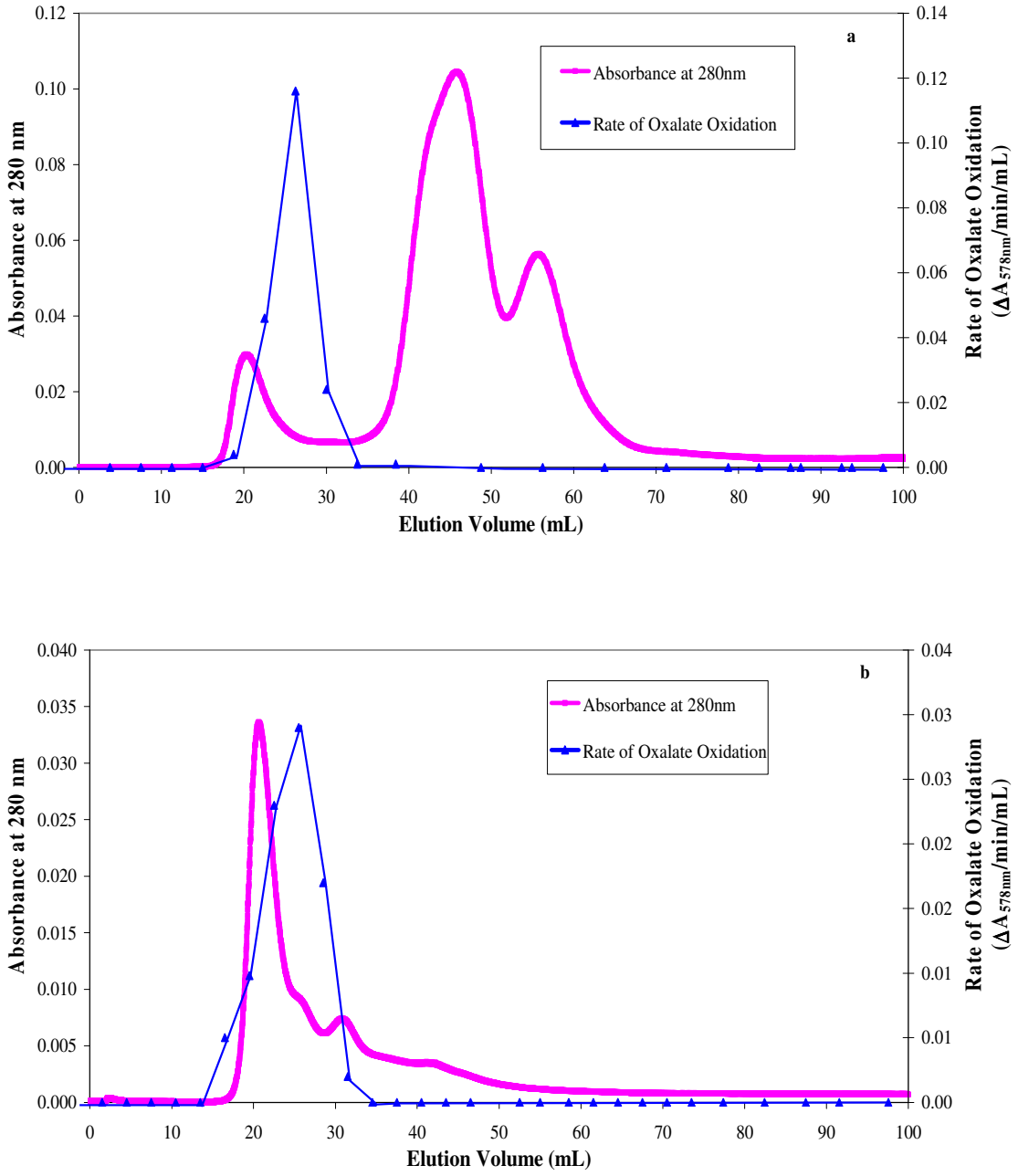


Figure 4.2: Sephacryl-S-200-HR Chromatography of Barley Root Oxalate Oxidase.

Active Oxox fractions after ASF (a) and cation exchange chromatography (b) were resolved using the Sephacryl S-200-HR column following method 2.11.2. Protein content at A_{280nm} was monitored online, a selection of eluted fractions (3 mL to 5 mL) were assayed using the MBTH assay method 2.12.6 at A_{578nm} in triplicate.

Purification of the crude ASF concentrated barley root extract in figure 4.2a led to the elution of additional peaks in comparison to the extract that had been additionally purified using ASF and cation exchange chromatography (figure 4.2b), indicating the latter extract was purer. It is visible from figure 4.2a and 4.2b that barley root Oxox eluted at similar

positions since Oxox activity was measured at approximately similar elution volumes.

The further purification of barley root Oxox after ASF (figure 4.2a) and cation exchange chromatography (figure 4.2b) using gel filtration chromatography led to the elution of approximately 89 % and 85 % of the total Oxox activity applied to the column respectively.

In conclusion, active barley root Oxox extracts after ASF and cation exchange column were successfully purified using gel filtration chromatography. However, the chromatography of the barley root extract after cation exchange chromatography led to the enhanced resolution of components.

4.6 OVERVIEW OF THE PURIFICATION OF OXALATE OXIDASE FROM BARLEY ROOTS

The purification of barley root Oxox was achieved using a five step purification protocol employing subcellular fractionation, thermal treatment, ASF, cation exchange chromatography and gel filtration chromatography. The protein content of the active Oxox extracts after each procedure was monitored and used to calculate the specific activity and the purification factor, shown in table 4.4.

	Specific Activity (μmol min^{-1} mg^{-1})	**Purification Factor**
Subcellular Fractionation (Crude Extract)	0.166	0
Thermal Treatment	0.918	5.5
Ammonium Sulphate Fractionation	1.871	11.3
Cation Exchange Chromatography	2.867	17.3
Size Exclusion Chromatography	9.534	57.4

Table 4.4: An overview of the Purification of Barley Root Oxalate Oxidase.

The specific activity of μmol min^{-1} mg^{-1} of protein represents Units per mg.

The purification of Oxox from barley roots led to an approximate 9 fold increase in the specific activity. The combination of purification procedures employed to purify barley root Oxox was very successful since Oxox was purified 57 fold during this research.

The increase in specific activities in table 4.4 shows similar trends to the data published by

numerous research groups [72-74, 78, 80, 82, 84], whereby increases in specific activity and purification fold were observed. The specific activities during this research were not as high as some published, a possible explanation for this may be due to the fungal attacks on barley roots during cultivation. Even though fungal attacks have been reported to increase Oxox activity in certain sources, the fungal infections incurred during this research were highly adverse. Therefore, an increase in activity was not measured, in contrast, barley root Oxox activity was decreased.

4.7 DEVELOPMENT OF A BIOSENSOR USING PURIFIED BARLEY ROOT OXALATE OXIDASE

Amperometric analysis was undertaken using each of the samples collected during purification. This was performed to determine the ability of barley root Oxox active extracts to detect oxalate since this research aims to detect oxalate amperometrically in patients suffering from primary hyperoxaluria (PH). These series of experiments would also identify whether the purity of Oxox was an essential requirement to detect oxalate.

Barley root extract purified after each purification procedure was immobilised onto a platinum electrode following method 2.18. Sequential additions of 0.1 mM oxalate were added during amperometric studies. Experiments were performed in triplicate and the resultant average biosensor response is shown in figure 4.3.

The immobilisation of the crude Oxox extract after the initial purification procedure of subcellular fractionation led to the least sensitive current response. An increase in the sensor response was observed after additional purification procedures were performed. As a result, the sensor immobilised with Oxox active fractions eluted during gel filtration chromatography was the most sensitive. Not only was this sensor more sensitive, it also possessed reduced error.

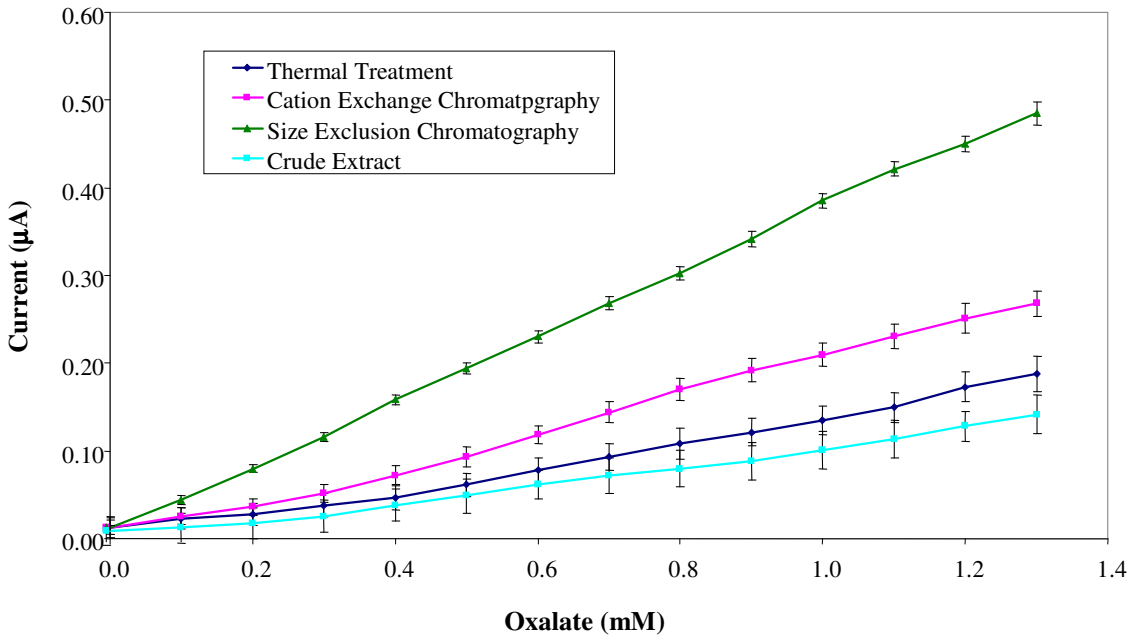


Figure 4.3: The Biosensor Response of Purified Barley Root Oxalate Oxidase.

The Oxox active fractions from each purification procedure were immobilised onto a platinum electrode following method 2.18. Sequential 0.1 mM additions of sodium oxalate were added and each experiment was performed in triplicate.

Amperometric analysis of the fractions collected during purification procedures possessed a linear range of 0 to 1.3 mM oxalate. This linear range exceeds that of the Sigma Oxox sensor developed in chapter 3.5.2 significantly, illustrating the successful development of a barley root Oxox biosensor.

4.8 CONCLUSION

The purification of Oxox from barley roots was achieved using a five step purification protocol employing subcellular fractionation, thermal treatment, ASF, cation exchange chromatography and gel filtration chromatography.

Oxox isolated from the soluble component of barley roots after subcellular fractionation possessed activity using both + and – oxalate assay mixtures. The – oxalate activity reduced to almost undetectable levels during the purification of Oxox.

Barley root Oxox was found to be thermally stable after exposing the extract to 60 °C for 10 minutes. To concentrate barley root Oxox, the thermally treated extract was subjected to 30 % to 65 % ASF. The ASF concentrated Oxox extract was purified further using

cation exchange chromatography and led to the elution of the desired enzyme at approximately 0.1 M sodium chloride. These findings were in accordance with those obtained by Requena and Bornemann during the purification of Oxox from barley roots [85].

Two sets of barley root Oxox extracts were purified using gel filtration chromatography, firstly, the ASF concentrated barley root Oxox extract and secondly, the Oxox active extract after ASF and cation exchange chromatography. The purification of both Oxox extracts led to the elution of Oxox at similar positions. However, unresolved separation of the ASF concentrated extract was observed during gel filtration chromatography.

Amperometric analysis undertaken with the extracts after each purification procedure illustrated the requirement to purify Oxox from barley roots since the sensor response observed increased as Oxox was purified further. Oxalate present in the urine of patients suffering from PH is conventionally three to six times higher than the normal level of 450 μM [7, 8], therefore, the Oxox sensor developed using Oxox active fractions after gel filtration chromatography can be used to diagnose PH sufferers effectively.

Having developed a working purification and amperometric protocol, an alternative novel source of Oxox was investigated. The presence of Oxox in additional crops from a number of families were investigated.

CHAPTER 5

SCREENING AND ISOLATION OF AN ALTERNATIVE SOURCE OF OXALATE OXIDASE

One of the aims of this research was to isolate a novel and alternative source of oxalate oxidase (Oxox). This involved the screening for Oxox in the soluble and insoluble components of sources families belonging to the *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Fabaceae*, *Lamiaceae*, *Poaceae* and *Solanaceae* families.

The potential sources selected during the screening process would be investigated further to determine a suitable and stable enzymatic assay. Alternative sources of Oxox will be purified employing thermal treatment, cation exchange chromatography and gel filtration chromatography.

5.1 SCREENING FOR OXALATE OXIDASE

Barley, maize, rye, sorghum and wheat are all members of the *Poaceae* family, all of which have been reported to contain Oxox, however, the location of the enzyme within each crop varies. Oxox purified from barley roots is located within the soluble components [24, 28, 44, 67, 69, 70, 72, 75, 78, 82-85, 87, 90, 95, 97, 104, 109, 122, 124, 126], this was confirmed by the studies performed in chapter 4.1. Oxox present in sorghum roots [126] and sorghum leaves [24, 28, 70, 72, 82, 83] have also been reported to be present within the soluble components, whereas Oxox in rye leaves [120], maize roots [79] and wheat seedlings [109] are insoluble, membrane bound. A selection of crops from this family: barley, rye, sorghum and two varieties of wheat: summer and winter were investigated as possible sources.

Other plant families screened were the *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Cucurbitaceae*, *Fabaceae*, *Lamiaceae*, *Liliaceae* and *Solanaceae* families. The *Apiaceae* family of vegetables are regarded as 'root' vegetables and were screened since Oxox has been reported and is well documented in barley roots. The *Brassicaceae* family was screened since thale cress [127] has been reported to contain Oxox, therefore, multiple 'green vegetables' from this family were investigated. It is not apparent from the literature whether the *Asteraceae*, *Cucurbitaceae*, *Fabaceae*, *Lamiaceae* and *Liliaceae* families have been screened to detect Oxox, as a result, a number of sources from these families were studied.

The *Poaceae* family of crops: barley, rye, sorghum, summer wheat and winter wheat were cultivated following method 2.4.1, whereas the remaining sources investigated were selected on the basis of commercial and cultivation availability.

Where possible, the outer skin, inner seeds, stems, roots, seedlings and leaves were investigated in addition to the 'plant' itself during the screening for Oxox. Potential sources screened from the *Poaceae* family were homogenised and subjected to subcellular fractionation following method 2.5.1, whereas all other sources were prepared according to method 2.5. Both the soluble (pellet) and insoluble (supernatant) components after subcellular fractionation were investigated. Insoluble membrane bound components were released by treating with sodium deoxycholate, following method 2.5.

The soluble and insoluble components of each 'plant' from the *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Lamiaceae*, *Fabaceae* and *Solanaceae* families were analysed using the ABTS assay. This assay was selected since large number of assays were performed during the screening process, therefore, this assay was the least labour intensive. Conversely, the soluble and insoluble components of the leaves, roots and seedlings of each *Poaceae* crop were assayed using the MBTH assay, since the MBTH assay was found to be the optimum assay for barley roots, a member of the *Poaceae* family. The ABTS and MBTH assays were prepared containing (+), and in the absence (–) of oxalate.

The – oxalate assays were undertaken to identify the 'background' activity since activity was measured using the – oxalate assay mixture during the purification of Oxox from barley roots in chapter 4.1. The resultant 'oxalate oxidase' and 'oxidase' activities measured after performing + and – oxalate assays respectively, were used to calculate the 'corrected rate' illustrating the true extent of the rate of oxalate oxidation within the sources. An overview of the sources screened are shown in table 5.1. The sources possessing activity during the screening process are shown in table 5.2.

Chinese cabbage leaves	Bean sprouts	Fennel stems	Melon
Chinese cabbage stems	Asparagus stems	Calabreses	Carrot
Winter wheat seedlings	Asparagus tops	Cauliflower	Celeriac
Broad bean leaves	Beetroot leaves	French bean	Celery
Scarlet runner bean peas	Aubergine peel	Broad bean	Kale (red)
Chinese chives flower	Bamboo shoots	Barley roots	Kohlrabi
Chinese chives	Barley seedlings	Barley leaves	Chicory
Chinese radish peel	Barley shoots	Head lettuce	Courgette
Chinese radish	Coriander leaves	Fennel leaves	Shallot
Singkwa towel gourd peel	Coriander stems	Lemon grass	Cucumber
Singkwa towel gourd	Sorghum leaves	Marrow peel	Lupin
Bitter cucumber inside	Sorghum roots	Day-lily root	Lily bulb
Bitter cucumber peel	Sorghum shoots	Day-lily	Dill stems
Bitter cucumber	Green radish	Chayote peel	Endive
Summer wheat seedlings	Broccoli florets	Chayote	Parsnip
Radish (red) leaves	Broccoli stems	Mint leaves	Rye roots
Pea leaves & stems	Brussel sprouts	Mint stems	Marrow
Winter squash peel	Cabbage (green)	Parsley stems	Sage
Winter wheat leaves	Cabbage (white)	Spinach beet	Salsify
Winter wheat roots	Marrow leaves	Radish (red)	Alfalfa
Scarlet runner bean	Spinach leaves	Radicchio	Artichoke
Scarlet runner bean leaves	White gourd peel	Red onion	Aubergine
Sugar snap pea leaves	Potato and peel	Rosemary	Garlic
Sugar snap pea stems	Potato stems	Runner bean	Leek
Sugar snap pea pods	Swede peel	Yam bean peel	Squash
Summer wheat leaves	Squash peel	Yam bean	Swede
Summer wheat roots	Tomato and peel	Sugar snap pea	Basil
Summer wheat shoots	Tomato stems	Swede leaves	Beetroot
Winter wheat shoots	Fenugreek stems	Sweetcorn	Quinoa
Runner bean leaves	Turnip leaves	Sugar cane	Pumpkin
White gourd inside	Turnip peel	Swiss chard	Pea
Sorghum seedlings	Winter squash	Tarragon	Pea pods
Green radish peel	Indian mustard	Kale (green)	Pepper
Carrot leaves	Courgette stem	Dill leaves	Thyme
White gourd seeds	Rye seedlings	Rye shoots	Turnip
Runner bean peas	Parsley leaves	Rye leaves	Sorrel

Table 5.1: Sources Screened for Oxalate Oxidase Activity.

Assays were performed using the ABTS assay at A_{650nm} and MBTH assay at A_{578nm} (*Poaceae* family) in triplicate following method 2.12.6.

		Rate of Oxalate Oxidative (ΔA/min/mL)		
		+ Oxalate	**- Oxalate**	**Corrected Rate**
***Poaceae* Family**				
Barley	Leaves	ND	0.032 ± 0.002	**ND**
	Roots	0.426 ± 0.001	0.078 ± 0.001	**0.348 ± 0.001** *
	Seedlings	0.043 ± 0.000	ND	**0.043 ± 0.000**
Rye	Leaves (insoluble)	0.011 ± 0.001	ND	**0.011± 0.001**
Summer Wheat	Roots (insoluble)	0.034 ± 0.008	ND	**0.034 ± 0.008**
	Seedlings (insoluble)	0.049 ± 0.011	0.013 ± 0.001	**0.036 ± 0.011**
Winter Wheat	Seedlings (insoluble)	0.021 ± 0.004	ND	**0.021 ± 0.004**
***Apiaceae* Family**				
Carrot Leaves		1.084 ± 0.134	0.442 ± 0.059	**0.642 ± 0.146** *
Coriander Leaves (insoluble)		0.030 ± 0.005	0.008 ± 0.001	**0.022 ± 0.005**
Dill Stems		0.117 ± 0.030	0.021 ± 0.005	**0.096 ± 0.304**
Parsley Leaves		0.342 ± 0.033	0.057 ± 0.008	**0.285 ± 0.034** *
***Asteraceae* Family**				
Head Lettuce		0.807 ± 0.031	0.386 ± 0.027	**0.421 ± 0.041** *
***Brassicaceae* Family**				
Cabbage - green		0.572 ± 0.055	0.057 ± 0.019	**0.515 ± 0.058** *
Cauliflower Florets		0.470 ± 0.062	0.016 ± 0.006	**0.454 ± 0.062** *
Chinese Cabbage Leaves		0.063 ± 0.000	0.016 ± 0.002	**0.047 ± 0.002**
Chinese Cabbage Leaves (insoluble)		0.011 ± 0.003	ND	**0.011 ± 0.003**
Chinese Cabbage Stems		0.091 ± 0.008	ND	**0.091 ± 0.008**
Indian Mustard		0.055 ± 0.007	ND	**0.045 ± 0.007**
***Fabaceae* Family**				
Scarlet Runner Bean		0.062 ± 0.012	0.021 ± 0.009	**0.039 ± 0.015**
Broad Bean Leaves		0.140 ± 0.032	0.128 ± 0.016	**0.012 ± 0.036**
Fenugreek Leaves		1.916 ± 0.061	1.589 ± 0.053	**0.327 ± 0.081** *
***Lamiaceae* Family**				
Mint Leaves		3.708 ± 0.174	2.871 ± 0.123	**0.837 ± 0.213** *
Thyme (insoluble)		0.179 ± 0.054	0.051 ± 0.024	**0.128 ± 0.003**

Table 5.2: Sources Possessing Oxalate Oxidase Activity.

The Oxox activities measured in the **soluble** and insoluble components are shown. Assays were performed using the ABTS assay at A_{650nm} and MBTH assay at A_{578nm} (*Poaceae* family) in triplicate following method 2.12.6. Rates below 0.010 ΔA/min/mL are denoted as ND 'none detected'. Potential sources of Oxox which were selected for further analysis are marked with *.

Screening for Oxox in the roots, seedlings and leaves of the crops from the *Poaceae* family led to the detection of Oxox activity in both the soluble and insoluble components. The soluble components of barley roots and seedlings possessed Oxox activity, whereas the insoluble components of rye leaves, summer wheat roots and seedlings, and winter wheat

seedlings contained Oxox activity. The presence of Oxox within these crops is in accordance with the data published by numerous research groups since Oxox has been isolated and purified from these sources [28, 44, 67, 70, 72, 76, 78, 82-86, 97, 104, 120, 122, 127, 126].

Assays performed with the sources from the *Apiaceae* family led to the detection of Oxox activity in the soluble components of carrot leaves, dill stems and parsley leaves, in addition to the insoluble component of coriander leaves. The Oxox activities measured varied, however, the corrected rates of carrot and parsley leaves were significantly higher resulting in 0.642 ΔA_{650nm}/min/mL and 0.285 ΔA_{650nm}/min/mL respectively.

The only plant from the *Asteraceae* family possessing Oxox activity was head lettuce leaves being 0.421 ΔA_{650nm}/min/mL. Very high activity was measured using both + and – oxalate ABTS assay mixtures.

Screening of the *Brassicaceae* family led to the detection of Oxox activity in a number of plants. The corrected rates for cabbage leaves and cauliflower florets of 0.515 ΔA_{650nm}/min/mL and 0.454 ΔA_{650nm}/min/mL respectively were relatively high in comparison to the activities measured in the other plants from this family.

The soluble components of three members of the *Fabaceae* family possessed significant activities. Scarlet runner bean, broad bean leaves and fenugreek leaves were found to contain Oxox, however, the highest rate of oxalate oxidation was found in fenugreek leaves of 0.327 ΔA_{650nm}/min/mL.

Two plants from the *Lamiaceae* family generated Oxox activity: the soluble component of mint leaves and the insoluble component of thyme. The Oxox activities measured in mint leaves using – oxalate ABTS assay mixture was approximately 77 % of the + Oxox activity. As a result, the corrected rate was low in comparison to the Oxox activity initially measured.

5.1.1 Overview of the Screening Process

A number of plants from the *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Cucurbitaceae*, *Fabaceae*, *Lamiaceae*, *Liliaceae*, *Poaceae* and *Solanaceae* families were screened to detect the presence of Oxox.

During the cultivation of the crops from the *Poaceae* family, problems were encountered whilst harvesting barley, rye, sorghum, summer wheat and winter wheat grains since they were highly susceptible to fungal infections despite following the published sterilisation procedures. Larger quantities were cultivated in various environments including propagators (method 2.4.1), growing chambers (method 2.4.2) and in the 'open atmosphere' in an attempt to combat the fungal infections. A number of cultivation media were also investigated, as it was thought the medium may be aiding the fungal infections, therefore, all grains were cultivated in aerated water, sand, compost and gravel independently. Despite the alternative cultivation mediums and environments, the fungal infections persisted. Since the fungal infections were detrimental to the crops from the *Poaceae* family coupled with the labour intensive cultivation procedure, the further analysis of potential Oxox in these crops were not pursued.

During the screening process, a number of sources possessed substantial Oxox activity using + oxalate assay mixture, however, the overall corrected activity did not reflect this since the – oxalate activity was proportionally higher in some sources. If the purification of Oxox was based on the activity measured using + oxalate assay mixture, this screening process would have led to the 'presence' of Oxox in countless sources. These studies illustrate that the – oxalate assays performed were vital in determining the 'true' Oxox activity.

It is unclear from the literature whether research groups performed – oxalate assays during the purification of Oxox from various sources, therefore, a true representation of the Oxox activity would not have been apparent. As a result, this research questions the reliability of the published sources of Oxox. These studies also demonstrate that certain families are not more likely to possess Oxox activity if the presence of the enzyme has been reported in one source from a single family, unlike the trends that occur in the *Poaceae* family.

Assays performed after subcellular fractionation with the soluble and insoluble components of each 'plant' led to the identification of Oxox in a number of possible sources. The screening process led to significantly higher corrected Oxox activities in the soluble components of these sources (indicated with an asterisk in table 5.2):

- Barley roots
- Carrot leaves
- Cauliflower florets
- Fenugreek leaves
- Green cabbage leaves
- Lettuce leaves
- Mint leaves
- Parsley leaves

Following the screening studies, plants with high corrected activity (> 0.250 ΔA/min/mL) were further analysed to determine an optimum assay and the stability of Oxox in these extracts.

5.2 ASSAY SELECTION

Prior to the purification of possible Oxox from the soluble components of the seven sources, it was essential to select an optimum enzymatic assay. Although the ABTS assay detected substantial Oxox activity within the seven sources during the screening process, this assay can not be regarded as the optimum assay. Therefore, these sources were also assayed using the aminophenazone and MBTH methods. These studies were not only undertaken to determine an optimum assay, but also to identify an assay which would reduce the background – oxalate activities.

The activities measured after assaying the potential sources of Oxox using both + and – oxalate ABTS, aminophenazone and MBTH assay mixtures are shown in table 5.3.

	Rate of Oxalate Oxidative (ΔA/min/mL)		
	+ Oxalate	**- Oxalate**	**Corrected Rate**
Cabbage Leaves			
ABTS	0.772 ± 0.055	0.157 ± 0.019	**0.615 ± 0.058**
Aminophenazone	0.313 ± 0.068	0.077 ± 0.023	**0.236 ± 0.072**
MBTH	0.529 ± 0.047	0.251 ± 0.013	**0.278 ± 0.049**
Carrot Leaves			
ABTS	1.084 ± 0.134	0.442 ± 0.059	**0.642 ± 0.146**
Aminophenazone	0.786 ± 0.126	0.457 ± 0.045	**0.329 ± 0.134**
MBTH	0.827 ± 0.156	0.401 ± 0.034	**0.426 ± 0.160**
Cauliflower Florets			
ABTS	0.470 ± 0.062	0.016 ± 0.006	**0.454 ± 0.063**
Aminophenazone	0.394 ± 0.067	0.022 ± 0.010	**0.372 ± 0.068**
MBTH	0.832 ± 0.056	0.223 ± 0.099	**0.609 ± 0.114**
Fenugreek Leaves			
ABTS	1.516 ± 0.061	1.189 ± 0.153	**0.327 ± 0.165**
Aminophenazone	0.935 ± 0.108	0.676 ± 0.167	**0.259 ± 0.199**
MBTH	2.114 ± 0.164	1.605 ± 0.173	**0.509 ± 0.238**
Lettuce Leaves			
ABTS	0.307 ± 0.031	0.286 ± 0.027	**0.021 ± 0.041**
Aminophenazone	0.783 ± 0.024	0.281± 0.033	**0.502 ± 0.041**
MBTH	0.564 ± 0.045	0.472 ± 0.052	**0.092 ± 0.069**
Mint Leaves			
ABTS	3.708 ± 0.174	1.871 ± 0.125	**1.837 ± 0.241**
Aminophenazone	1.248 ± 0.138	0.442 ± 0.142	**0.806 ± 0.198**
MBTH	2.551 ± 0.136	1.826 ± 0.130	**0.725 ± 0.188**
Parsley Leaves			
ABTS	0.342 ± 0.033	0.057 ± 0.008	**0.285 ± 0.034**
Aminophenazone	0.371 ± 0.041	0.260 ± 0.013	**0.111 ± 0.043**
MBTH	0.676 ± 0.026	0.316 ± 0.005	**0.360 ± 0.026**

Table 5.3: The Activity of Oxalate Oxidase in High Activity Plant Sources using Different Assays.

Oxox activity was monitored at A_{650nm}, A_{520nm} and A_{578nm} for the ABTS, aminophenazone and MBTH assays respectively. All assays were performed following method 2.12.6 in triplicate, N=3.

The assays performed with cabbage, carrot, fenugreek, lettuce, mint, parsley leaves and cauliflower florets using the ABTS, aminophenazone and MBTH assays led to repeatable and reproducible results. Assaying lettuce leaves using the aminophenazone assay resulted in the highest corrected rate of oxalate oxidation in comparison to the ABTS and MBTH assays.

Optimum enzymatic assay studies performed with cabbage, carrot and mint leaves led to elevated corrected rates after assaying using the ABTS assay in comparison to those generated with the aminophenazone and MBTH assays.

Assays performed with cauliflower florets, fenugreek and parsley leaves led to high corrected oxalate oxidative rates using the MBTH assay. However, the oxidase activity measured using the – oxalate MBTH assay mixture was greater in comparison to the ABTS and aminophenazone assays.

In conclusion, the aminophenazone assay detected higher Oxox activities in the soluble component of lettuce leaves, therefore, this assay will be employed to further study Oxox from this source. The ABTS assay was used from this point on to measure Oxox activity in the soluble components of cabbage, carrot and mint leaves. Having established the MBTH assay was found to be the optimum assay for cauliflower florets, fenugreek and parsley leaves, this assay was employed during the purification of Oxox from these sources.

5.3 STABILITY STUDIES

The stability of Oxox within cabbage, carrot, fenugreek, lettuce, mint, parsley leaves and cauliflower florets was investigated prior to performing further purification procedures since instability (chapters 3.4.1.2.2 and 3.4.1.3) and insolubility (chapter 3.4.2.1) issues arose during the purification of Sigma Oxox. Since the isolated enzyme will be stored at -20 ºC until further use, it was vital to investigated whether Oxox was stable after storing at -20 ºC for up to 10 days. If Oxox is found to be unstable, particular sources would not be investigated further since a suitable amperometric sensor with a long shelf life would not be developed.

The seven potential sources of Oxox were homogenised in parallel following method 2.5 and stored at -20 ºC. The extracts were assayed on day 0, 5 and 10 using the optimum assays determined in chapter 5.2.

Oxox in cabbage, carrot and mint leaves was found to be stable after storing at -20 ºC for 10 days (results not shown). Conversely, fenugreek, lettuce, parsley leaves and

cauliflower florets Oxox were found not to be stable at -20 ºC up to 10 days. The resultant Oxox activities of the latter four Oxox sources are shown in table 5.4.

	Rate of Oxalate Oxidation (ΔA_{nm}/min/mL)		
	Day 0	**Day 5**	**Day 10**
Cauliflower Florets	0.770 ± 0.094	0.594 ± 0.057	0.531 ± 0.043
Fenugreek Leaves	2.016 ± 0.161	1.972 ± 0.178	1.674 ± 0.119
Lettuce Leaves	0.671 ± 0.051	0.243 ± 0.030	0.126 ± 0.009
Parsley Leaves	0.687 ± 0.033	0.595 ± 0.016	0.253 ± 0.040

Table 5.4: The Stability of Oxalate Oxidase in Fenugreek, Lettuce, Parsley Leaves and Cauliflower Florets.

Assays were conducted using the optimum aminophenazone and MBTH assays at A_{550nm} and A_{578nm} (+ oxalate only) in triplicate following method 2.12.6, N=3.

The stability studies performed with fenugreek, lettuce, parsley leaves and cauliflower florets led to significant reductions in Oxox activity after storing the extracts at -20 ºC for up to 10 days. Lettuce leaves Oxox was the most unstable since 81 % of its activity was reduced after 10 days. Storing cauliflower florets and parsley leaves Oxox during this period at -20 ºC led to the reduction of approximately 31 % and 63 % Oxox activity respectively. Only 17 % of the Oxox activity was reduced after storing fenugreek leaves for 10 days, however, this loss was substantially high being approximately 0.342 ΔA_{578nm}/min/mL.

In conclusion, storing fenugreek, lettuce, parsley leaves and cauliflower florets up to 10 days at -20 ºC led to a reduction in the Oxox activity measured, illustrating the instability of possible Oxox within these sources.

Having identified Oxox from fenugreek, lettuce leaves and cauliflower florets were not stable, their purification was not pursued. Hence, the purification of possible Oxox from the soluble components of cabbage, carrot and mint leaves was undertaken.

Prior to the purification of Oxox from these extracts, the variability of the Oxox activities after preparing cabbage and mint leaves from different varieties and sources of origin was investigated.

5.4 VARIATION OF OXALATE OXIDASE ACTIVITY IN MINT AND CABBAGE LEAF SOURCES

Prior to purifying Oxox from cabbage, carrot and mint leaves, an issue needed to be addressed regarding the variation in Oxox activities measured in cabbage and mint leaves on different occasions. To investigate this, cabbage and mint leaves were cultivated, in addition to being purchased from commercial suppliers.

Two varieties of cabbage leaves were cultivated independently, the varieties of which were: garden cabbage (*Brassica oleacea var. capitata*) and Chinese cabbage (*Brassica rapa var. pekinensis*). The cultivated cabbage leaves were investigated simultaneously alongside commercially available cabbage from two different sources of origin: Israeli and Spanish. Mint leaves were investigated in a similar manner, however, three varieties were cultivated, apple (*Mentha suaveolens*), basil (*Mentha spp*.) and chocolate (*Mentha piperita cv*.) in addition to commercially available British, Columbian, Israeli and Spanish mint leaves. The cultivated and purchased cabbage and mint leaves were homogenised and subjected to subcellular fractionation, after which they were assayed using + and – oxalate assay mixtures, shown in table 5.5a (cabbage leaves) and 5.5b (mint leaves).

	Rate of Oxalate Oxidation (ΔA_{650nm}/min/mL)		
Variety	**+ Oxalate**	**- Oxalate**	**Corrected Rate**
Garden Cabbage	0.782 ± 0.049	0.160 ± 0.045	**0.622 ± 0.067**
Chinese Cabbage	0.081 ± 0.026	0.033 ± 0.019	**0.048 ± 0.032**

	Rate of Oxalate Oxidation (ΔA_{650nm}/min/mL)		
Origin	**+ Oxalate**	**- Oxalate**	**Corrected Rate**
Israel	0.046 ± 0.016	0.022 ± 0.009	**0.024 ± 0.018**
Spain	0.094 ± 0.026	0.061 ± 0.030	**0.033 ± 0.040**

Table 5.5a: The Rate of Oxalate Oxidation of Cultivated and Commercially Available Cabbage Leaves.

Oxox activity in the soluble components of garden and Chinese cabbage varieties, and commercially available Israeli and Spanish cabbage leaves. All assays were performed in triplicate using the ABTS assay at A_{650nm} following method 2.12.6, N=3.

	Rate of Oxalate Oxidation (ΔA_{650nm}/min/mL)		
Variety	**+ Oxalate**	**- Oxalate**	**Corrected Rate**
Apple Mint	0.085 ± 0.021	0.067 ± 0.024	**0.018 ± 0.032**
Basil Mint	0.024 ± 0.006	0.018 ± 0.008	**0.006 ± 0.010**
Chocolate Mint	0.079 ± 0.034	0.067 ± 0.029	**0.012 ± 0.045**

	Rate of Oxalate Oxidation (ΔA_{650nm}/min/mL)		
Origin	**+ Oxalate**	**- Oxalate**	**Corrected Rate**
Britain	0.089 ± 0.033	0.078 ± 0.036	**0.011 ± 0.049**
Columbia	0.069 ± 0.029	0.032 ± 0.016	**0.037 ± 0.033**
Israel	2.914 ± 0.167	2.796 ± 0.121	**0.118 ± 0.206**
Spain	3.708 ± 0.174	1.871 ± 0.125	**1.837 ± 0.214**

Table 5.5b: The Rate of Oxalate Oxidation of Cultivated and Commercially Available Mint Leaves.

Oxox activity in the soluble components of apple, basil and chocolate mint varieties, and commercially available British, Columbian, Israeli and Spanish mint leaves. All assays were performed in triplicate using the ABTS assay at A_{650nm} following method 2.12.6.

Cultivated and purchased cabbage and mint leaves possessed varying Oxox and 'oxidase' activities. Spanish, Israeli and cultivated Chinese cabbage leaves contained insignificant Oxox activities, in comparison to cultivated garden cabbage leaves.

Assays performed with the cultivated apple, basil and chocolate mint leaves led to insignificant corrected rates, similarly, British and Columbian mint did not possess high corrected rates. Conversely, Israeli and Spanish mint leaves contained high activities using both + and – oxalate assay mixtures, however, the corrected oxalate oxidative rate of Spanish mint leaves was significantly higher than that of Israeli mint.

Differences in Oxox activity measured in cultivated and commercially available cabbage and mint leaves may be due to the conditions in which they were cultivated. Oxox has been reported in plants as a defence mechanism against fungal attacks, therefore, the sources possessing high Oxox activity may have possibly been exposed to, or were more susceptible to fungal infections in comparison to the sources possessing low activities.

These studies demonstrate that the source of a particular plant can effect the levels of Oxox present which may depend upon its origin, cultivation medium, variety and the likelihood of it being prone to fungal attacks.

The purification of Oxox from the soluble components of Spanish mint leaves and garden cabbage leaves in addition to carrot leaves was pursued by thermal treatment.

5.5 THERMAL TREATMENT

To assess whether Oxox in the soluble components of cabbage leaves (garden cabbage), carrot leaves and mint leaves (Spanish) were thermally stable, thermal treatment was performed. Assays were performed prior to and after thermal treatment at 60 °C for 10 minutes (method 2.6) using both + and – oxalate ABTS assay mixtures, the results of which are illustrated in table 5.6.

	Rate of Oxalate Oxidation (ΔA_{650nm}/min/mL)		
	+ Oxalate	**- Oxalate**	**Corrected Rate**
Mint Leaves			
Prior to Thermal Treatment	4.074 ± 0.180	2.630 ± 0.131	**1.916 ± 0.223**
After Thermal Treatment	3.564 ± 0.163	2.158 ± 0.151	**0.934 ± 0.222**
Cabbage Leaves			
Prior to Thermal Treatment	0.719 ± 0.073	0.173 ± 0.025	**0.546 ± 0.077**
After Thermal Treatment	0.520 ± 0.064	0.053 ± 0.017	**0.467 ± 0.066**
Carrot Leaves			
Prior to Thermal Treatment	1.125 ± 0.114	0.510 ± 0.047	**0.615 ± 0.123**
After Thermal Treatment	0.864 ± 0.075	0.319 ± 0.033	**0.545 ± 0.082**

Table 5.6: The Effect of Thermal Treatment on Oxalate Oxidase in Cabbage, Carrot and Mint leaves.

The soluble components of cabbage, carrot and mint leaves were thermally treated at 60 °C for 10 minutes following method 2.6. Enzymatic assays were performed using the ABTS assay at A_{650nm} in triplicate following method 2.12.6, N=3.

Thermally treating cabbage, carrot and mint leaves led to a reduction in the measured activity using + and – oxalate assay mixtures. This decrease in activity was due to the possible elimination of interfering species within the extracts which may have been interacting with the dye precursor and/or horseradish peroxidase (HRP) causing a change in the intensity of the coloured dye.

In conclusion, thermal treatment of cabbage, carrot and mint leaves demonstrated that Oxox within these sources were stable up to 60 °C for 10 minutes. These findings are in accordance with the previous studies performed in chapter 4.2, in addition to the published data by Kotsira and Clonis [78] and Requena and Bornemann [85] who suggested Oxox purified from barley root was stable under the same conditions.

To continue with the purification of Oxox in cabbage, carrot and mint leaves, the extracts were subjected to ammonium sulphate fractionation.

5.6 AMMONIUM SULPHATE FRACTIONATION

Thermally treated cabbage, carrot and mint leaves Oxox were subjected to ammonium sulphate fractionation (ASF), which not only concentrates, but was also expected to purify the enzyme of interest. Differing concentration of ammonium sulphate [24, 26-28, 67, 70, 72-74, 76, 80, 82-84, 120, 126] have been employed by research groups to concentrate and purify Oxox from varying sources.

Thermally treated Oxox in cabbage, carrot and mint leaves were subjected to 60 % ASF in 20 % increments to establish the optimum conditions for this procedure. After ASF, all extracts were dialysed following method 2.8 to remove residual ammonium sulphate from the samples.

ASF and dialysis of the cabbage, carrot and mint extracts led to almost complete loss of Oxox activity (results not shown). Total recovery of the extract was not obtained after concentrating the extract using 20 %, 40 % and 60 % ASF. As the Oxox activity in cabbage, carrot and mint leaves were sufficiently high in the thermally treated extracts, these samples were used during further purification using cation exchange chromatography.

5.7 CATION EXCHANGE CHROMATOGRAPHY

Cation exchange chromatography was employed and standardised during the purification of Sigma Oxox (chapter 3.4.1) and barley root Oxox (chapter 4.4). This standardised procedure was used to purify Oxox from cabbage, carrot and mint leaves. Prior to

performing this procedure, the stability of Oxox within these sources in sodium chloride was investigated, since this procedure is dependant upon the use of this salt. The stability of Oxox in sodium chloride was determined in a similar manner to previous studies in chapter 4.4 where varying concentrations of sodium chloride were added to each extract. Since Sigma and barley root Oxox eluted at approximately 0.1 M sodium chloride, the stability studies undertaken did not exceed 0.5 M. Assays were conducted immediately after the addition of the salt solution (0 to 0.5 M) and after storing the extracts at 4 °C for 24 hours. The assays performed illustrated Oxox within these sources were not significantly affected by up to 0.5 M sodium chloride (results not shown).

Once the stability of Oxox in sodium chloride was established, its purification using carboxymethyl (CM) cellulose cation exchange media was undertaken. Thermally treated extracts were assayed prior to purification to identify if the total activity applied to the column corresponded to the total activity eluted. Spanish mint leaves Oxox were purified with the same cation exchange column used during the purification of barley root Oxox following method 2.9.1.1. However, Oxox in cabbage and carrot leaves were purified using a smaller cation exchange column following method 2.9.1.2. Figure 5.1a-c illustrates the chromatograms obtained after the purification of Oxox from mint, cabbage and carrot leaves using cation exchange chromatography.

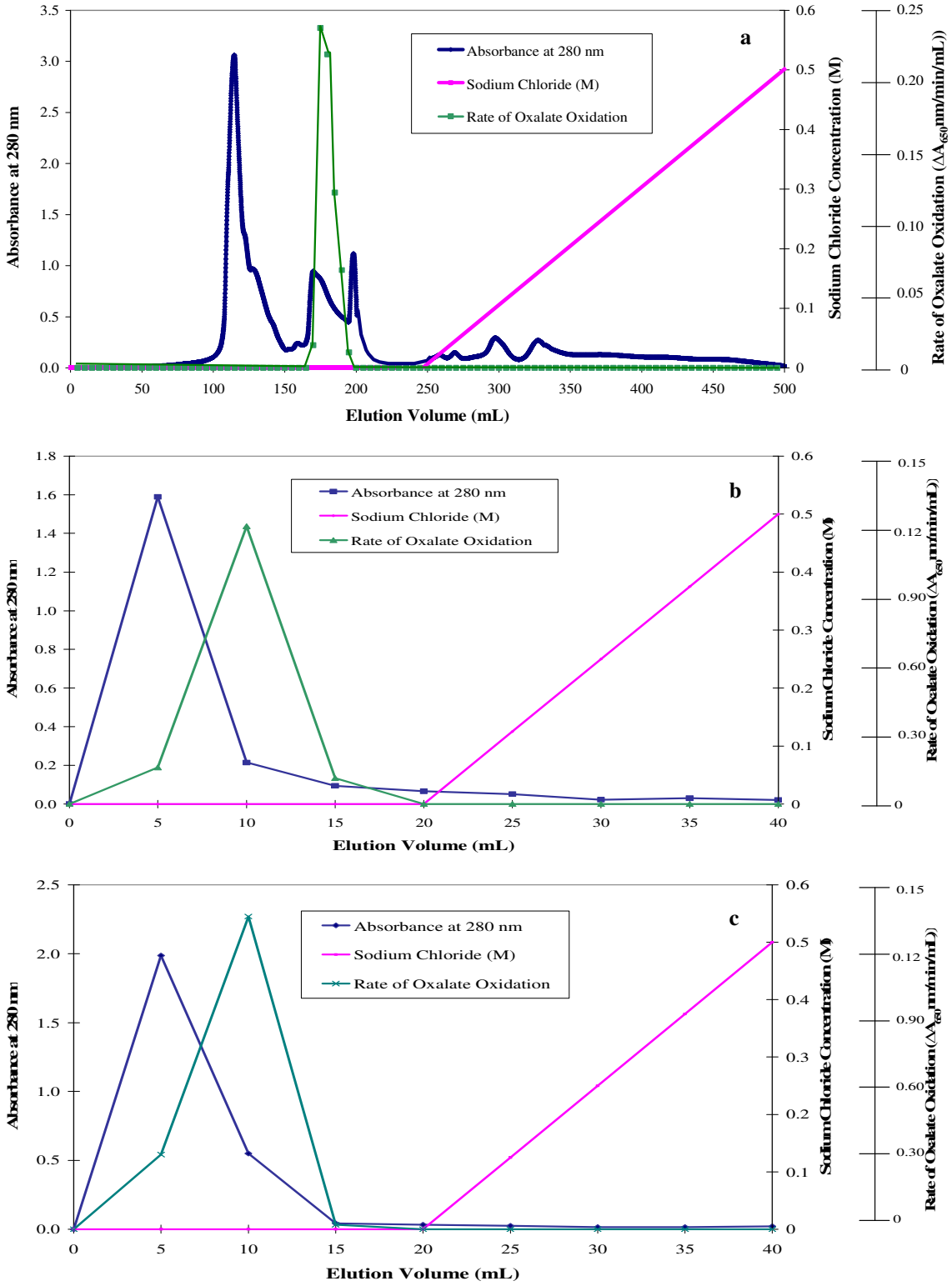


Figure 5.1: Cation Exchange Chromatography of Thermally Treated Mint, Cabbage and Carrot Leaves Oxalate Oxidase.

Oxox in thermally treated Spanish mint leaves (a), cabbage (b) and carrot (c) leaves were purified using the CM cellulose cation exchange following methods 2.9.1.1 and 2.9.1.2. Conductivity and protein content at A_{280nm} were monitored online. All eluted fractions (5 mL) were assayed in triplicate using ABTS assay at A_{650nm} following method 2.12.6.

Oxox activity was not measured in the fractions eluted during the salt gradients, however, the activity was detected in the components eluting prior to the addition of sodium chloride. Similar results were observed after the purification of Oxox from mint, cabbage and carrot leaves, where Oxox eluted prior to the introduction of sodium chloride. The percentage yield after the purification of Oxox using cation exchange chromatography corresponded to approximately 82 % ± 4.3 (mint leaves), 80 % ± 5.6 (cabbage leaves) and 79 % ± 7.1 (carrot leaves).

The results obtained after performing cation exchange chromatography with each of the novel sources were similar, however, they were not consistent with the findings in previous studies (3.4.1 and 4.4). The elution of Oxox prior to the introduction of the sodium chloride gradient may be due to two possible factors. Firstly, the component of interest, Oxox, may possess a negative charge or, secondly, it possesses a weak positive charge. These factors may have contributed to the inability of Oxox to interact with the negatively charged CM groups within the CM cellulose cation exchange media at pH 5.0.

To investigate whether Oxox in cabbage, carrot and mint leaves were positively charged, anion exchange chromatography was performed.

5.8 ANION EXCHANGE CHROMATOGRAPHY

Anion exchange chromatography has been employed by a number of research groups [24, 27, 28, 70, 72, 74, 75, 77, 80, 82] to purify Oxox from different sources. Diethylaminoethyl (DEAE) cellulose anion exchange media was used to determine if Oxox in the three sources possessed strong negatively charged groups, if so, they would bind to the DEAE groups within the anion media. This procedure has been employed not only to purify proteins, but also to purify small molecular mass components such as urate [141].

Thermally treated (60 °C for 10 minutes) cabbage, carrot and mint extracts were assayed prior to performing anion exchange chromatography following method 2.9.2, in addition to all eluted fractions. Anion exchange chromatography undertaken led to the detection of Oxox activity in the unbound fractions (results not shown), hence Oxox within the three sources did not interact with the DEAE groups within the anion exchanger.

The purification of Oxox in cabbage, carrot and mint leaves by anion and cation exchange chromatography led to similar findings, whereby, Oxox did not bind and interact with the charged groups on the anion and cation exchangers. As a result, it can be concluded that Oxox in the three sources are not strongly negatively or positively charged.

Considering Oxox from cabbage, carrot and mint leaves were exhibiting dissimilar ionic properties to barley root Oxox, an approximate molecular mass of cabbage leaves Oxox was determined using Sephacryl S-200-HR gel filtration chromatography.

5.9 GEL FILTRATION CHROMATOGRAPHY

Due to the lack of availability of commercial carrot and mint leaves, it was not possible to perform gel filtration chromatography using these two sources. Therefore, an approximate molecular mass of Oxox in cabbage leaves was determined using Sephacryl S-200-HR gel filtration chromatography. This media is commonly used to determine the molecular mass of many proteins and has also been employed by Whittaker and Whittaker to purify Oxox from barley roots [77].

The Oxox active extract eluted during cation exchange chromatography possessed low activity (0.241 ΔA_{650nm}/min/mL) in comparison to the thermally treated extract (3.564 ΔA_{650nm}/min/mL), as a result, the thermally treated cabbage extract was subjected to gel filtration chromatography following method 2.11.1.1. The extracts applied and eluted from the column were assayed to calculate the percentage yields, the resultant chromatogram obtained during these series of experiments is shown in figure 5.2.

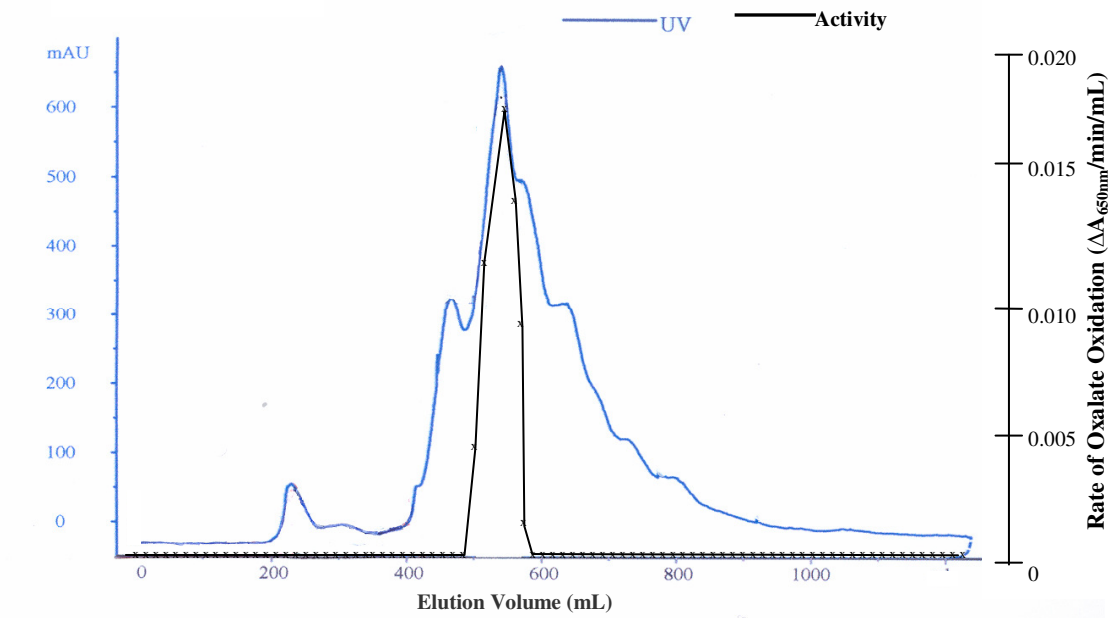


Figure 5.2: Sephacryl S-200-HR Chromatography of Oxalate Oxidase in Thermally Treated Cabbage Leaves.

Thermally treated cabbage leaves Oxox was subjected to gel filtration chromatography following method 2.11.1.1. All fractions eluted (10 mL) were assayed using the ABTS assay at A_{650nm} in triplicate following method 2.12.6.

The purification of Oxox in thermally treated cabbage leaves using Sephacryl S-200-HR chromatography led to the elution of approximately 87 % ± 6.3 of the activity applied to the column. The smallest molecular mass marker, Cytochrome c (12.4 kDa) eluted after 360 mL (figure 2.1), whereas cabbage Oxox eluted from the same column between 510 mL and 560 mL. The elution of Oxox after such a large volume indicated cabbage leaves Oxox was substantiality less than 12.4 kDa. Since the component of interest was found to be less than 12.4 kDa, these studies highlighted the difference in molecular mass between cabbage leaves Oxox and other purified Oxox's in table 1.10.

5.10 CONCLUSION

Plants from a number of families: *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Fabaceae*, *Lamiaceae*, *Poaceae* and *Solanaceae* were screened to identify the presence of Oxox. The screening process led to the detection of relatively high corrected Oxox activities in the soluble components of cabbage, carrot, fenugreek, lettuce, mint, parsley leaves and cauliflower florets. Optimum enzymatic assays and the stability of Oxox in the

seven sources over 10 days was determined prior to performing further purification procedures.

The optimum assay studies performed were successful and led to repeatable and reproducible Oxox activities using the ABTS, aminophenazone and MBTH assays. On the other hand, the stability studies undertaken led to unconventional findings. Cabbage, carrot and mint leaves Oxox were found to be stable over 10 days at 4 °C. Conversely, stability studies performed with lettuce, parsley, fenugreek leaves and cauliflower florets demonstrated Oxox within these sources were unstable. The instability of Oxox within the latter four sources over 10 days is problematic since firstly, the amperometric sensor to be developed must possess a long shelf-life and secondly, these extracts may be stored for this or prolonged durations during the purification of Oxox. As a result, the purification of Oxox from lettuce, parsley, fenugreek leaves and cauliflower florets was not continued.

The purification of cabbage, carrot and mint leaves was further pursued by thermal treatment by exposing the extracts to 60 °C for 10 minutes. Oxox in these extracts was found to be unaffected, illustrating the thermal stability of Oxox in cabbage, carrot and mint leaves.

Oxox in cabbage, carrot and mint leaves was concentrated and purified using 20 %, 40 % and 60 % ASF to determine the optimum conditions for this procedure. Negligible activities remained after undertaking ASF and dialysis, therefore, this purification procedure was omitted.

Thermally treated cabbage, carrot and mint leaves Oxox were purified further using cation and anion exchange chromatography. Oxox in these sources did not bind to the CM or DEAE groups on the cation and anion exchangers respectively, indicating they possess dissimilar isoelectric points to barley Oxox (chapter 4.4). The findings obtained were in contrast to those published where Oxox was purified using both cation exchange chromatography [69, 75, 77, 85, 114] and anion exchange chromatography [24, 27, 28, 70, 72, 74-77, 80, 82, 97, 103, 106, 122].

Cabbage leaf Oxox was later subjected to gel filtration chromatography. Sephacryl S-200-HR chromatography of cabbage leaves Oxox led to the elution of Oxox between 510 mL to 560 mL, whereas the smallest molecular mass marker, Cytochrome c with a molecular mass of 12.4 kDa eluted after 360 mL. The elution of cabbage leaves Oxox after such a large volume indicated it was much smaller than 12.4 kDa. These findings coincide with those obtained during ASF and dialysis where negligible activities were measured after these procedures. The dialysis tubing used possessed a molecular mass cut off of 12 kDa, therefore, the components of interest may have dialysed out of the membrane.

Since the molecular mass of cabbage leaves Oxox was dissimilar to the molecular masses of purified Oxox's (chapter 1.6.1), these findings bring into question whether the enzymes isolated from cabbage, carrot and mint leaves are in fact the same as Oxox isolated from barley roots.

In conclusion, Oxox in the soluble components of cabbage, carrot and mint leaves were thermally stable. The purification of Oxox from these extracts using cation exchange and size exclusion chromatography demonstrated Oxox exhibited dissimilar properties to barley root and other purified Oxox's. The elution of cabbage leaves Oxox outside the molecular marker of 12.4 kDa demonstrated the oxalate oxidising components initially thought to be Oxox, may not be.

Further studies were undertaken to characterise the oxalate oxidative components in cabbage, carrot and mint leaves, discussed in the subsequent chapter.

CHAPTER 6

CHARACTERISATION OF THE OXALATE OXIDISING COMPONENTS

The primary aim of this research, outlined in chapter 1.3, was to isolate and purify an alternative source of oxalate oxidase (Oxox), this has proven to be more challenging than expected. The purification of Oxox from the soluble components of garden variety cabbage leaves, carrot leaves and Spanish mint leaves demonstrated that the oxalate oxidative components isolated from these sources possessed dissimilar ionic and molecular properties to known Oxoxs.

This chapter highlights the studies undertaken to characterise the oxalate oxidative components isolated from the three sources. Gel filtration chromatography, dialysis, protein electrophoresis and thermal treatment were undertaken to enhance the knowledge of the oxalate oxidative components. Further analytical studies such as mass spectrometry and spectroscopy were also carried out in an attempt to identify the active components in cabbage and mint leaves.

6.1 THE MOLECULAR MASS OF THE OXALATE OXIDATIVE COMPONENTS

Gel filtration chromatography using Sephacryl S-200-HR (chapter 5.9) demonstrated the oxidative component in cabbage leaves was smaller than the molecular marker Cytochrome c of 12.4 kDa. As a result, studies were undertaken to investigate the approximate molecular mass of the oxalate oxidising components in cabbage, carrot and mint leaves.

6.1.1 Sephadex Gel Filtration Chromatography of Plant Extracts

To determine the molecular mass of the oxalate oxidative components in cabbage, carrot and mint leaves, Sephadex gel filtration chromatography was performed. The Sephadex G25 column was standardised with bovine serum albumin (BSA) containing 1 M sodium chloride to demonstrate the 'group separation' of components, shown in figure 2.2 in chapter 2.11.3. Sephadex G25 media possesses a fractionation range of 5 kDa, therefore, components with a molecular mass greater than 5 kDa elute in the initial 'protein peak' followed by smaller molecular mass components less than 5 kDa in the second 'salt peak'.

Thermally treated (60 °C for 10 minutes) cabbage, carrot and mint leaves oxalate oxidative components were subjected to Sephadex G25 chromatography using the standardised column following method 2.11.4. Eluted fractions were assayed using + and – oxalate

ABTS assay mixtures, the chromatograms after performing Sephadex G25 chromatography are shown in figure 6.1.

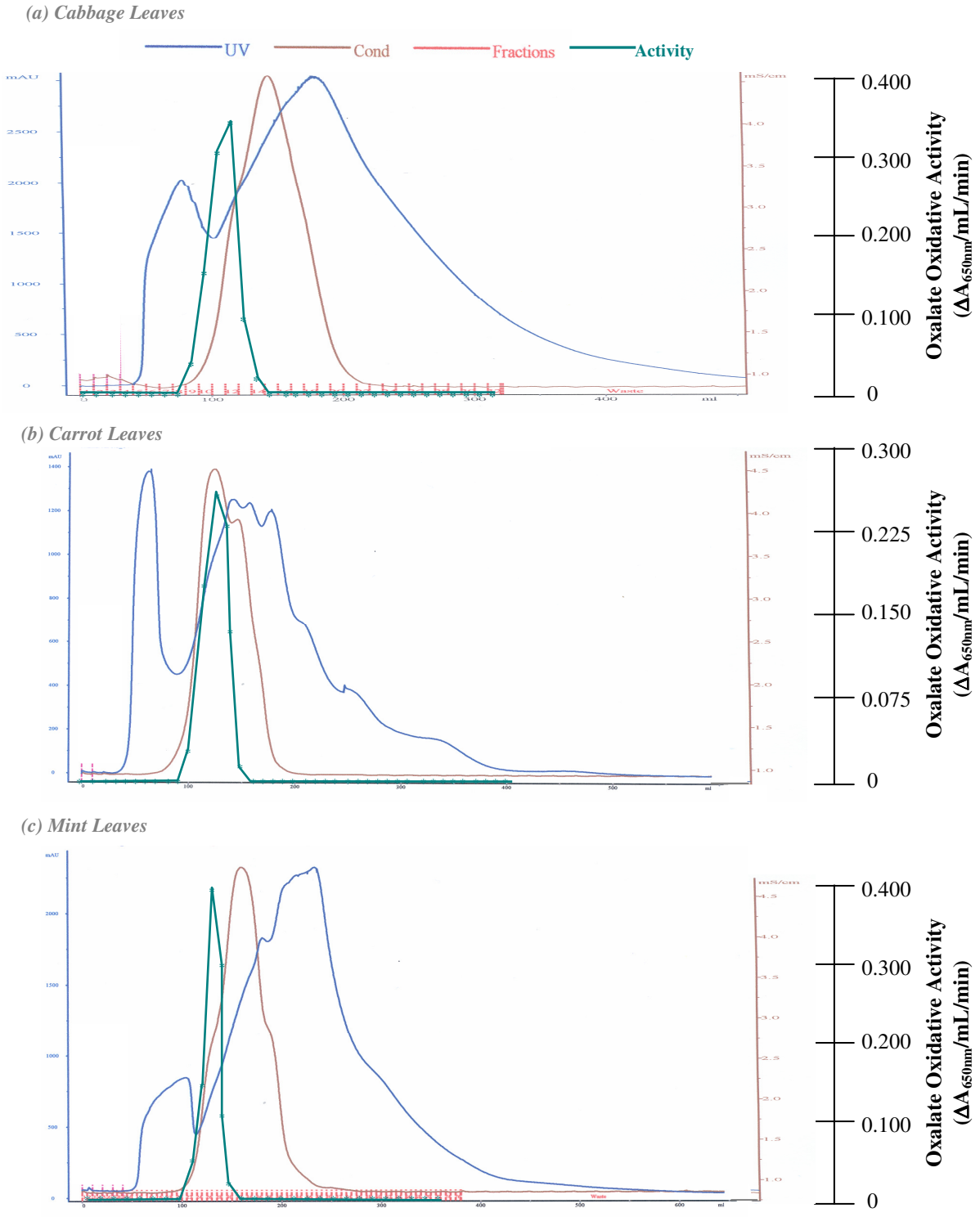


Figure 6.1: Sephadex G25 Chromatography of Cabbage, Carrot and Mint Leaves Oxalate Oxidising Components.

Thermally treated cabbage (a), carrot (b) and mint (c) leaves oxalate oxidising components were purified using Sephadex G25 chromatography, following method 2.11.4. Eluted fractions were assayed in triplicate using the ABTS assay method 2.12.6 at A_{650nm}.

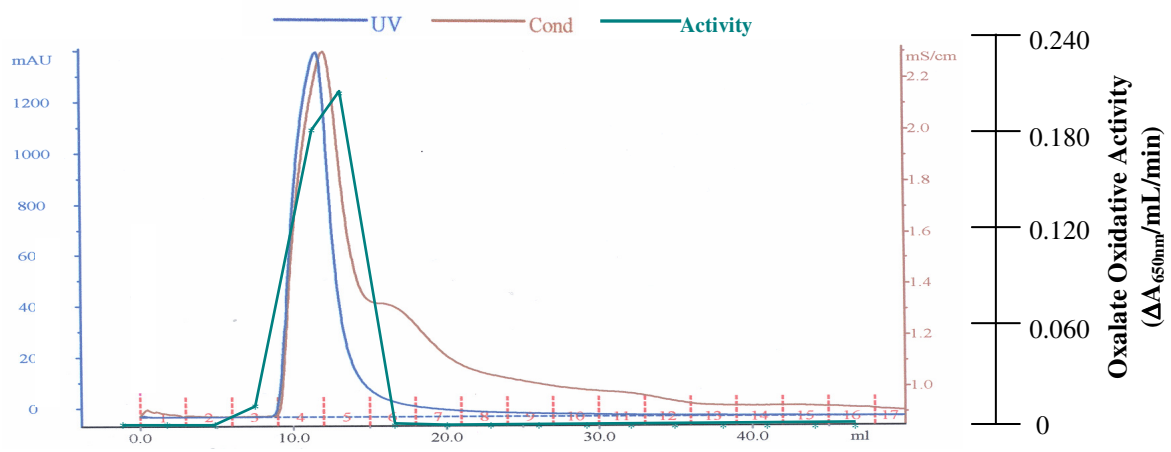
The crude purification of leaves from cabbage (figure 6.1a), carrot (figure 6.1b) and mint (figure 6.1c), oxalate oxidising components using Sephadex G25 chromatography resulted in the elution of a 'protein peak' and a 'salt peak', similar to the separation obtained in figure 2.2 with BSA and sodium chloride. The analysis of the eluted components did not lead to the detection of oxalate oxidative activity within the initial A_{280nm} absorbing material, therefore indicating the components in the three sources were smaller than 5 kDa.

Since all three extracts exhibited comparable Sephadex G25 profiles, only the active cabbage and mint oxalate oxidative extracts eluted during Sephadex G25 chromatography were subjected to further separation due to their availability. These extracts were applied to the Sephadex G15 and G10 columns to provide further resolution of the oxalate oxidising components following method 2.11.4. Sephadex G15 and G10 media employ similar principles to Sephadex G25, however, their fractionation ranges are equal to or less than 1 kDa and 700 Da respectively.

All eluted fractions collected during these studies were assayed using + and – oxalate ABTS assay mixtures, in addition to the extracts applied to the column. Figures 6.2 and 6.3 show the chromatograms of the oxalate oxidising components in cabbage and mint leaves respectively using Sephadex G15 and G10 gel filtration chromatography.

The active cabbage and mint fractions collected during Sephadex G25 chromatography were successfully resolved further using Sephadex G15 and G10 chromatography. The purification of the active Sephadex G25 oxalate oxidising components using Sephadex G15 (figures 6.2a and 6.3a), led to the elution of only one distinctive A_{280nm} absorbing peak which coeluted with the salt peak, as shown by the conductivity. Further purification of the active Sephadex G25 extracts using Sephadex G10 chromatography (figures 6.2b and 6.3b) resulted in the elution of two A_{280nm} peaks, and two salt peaks. After performing enzymatic assays on all the eluted fractions, the oxalate oxidising components were detected within the salt peaks during both Sephadex G15 and G10 chromatography. Enhanced resolution of the active Sephadex G25 extracts was obtained using Sephadex G10 column, rather than after Sephadex G15, as shown in figures 6.2 and 6.3.

(a) Sephadex G15 Chromatography



(b) Sephadex G10 Chromatography

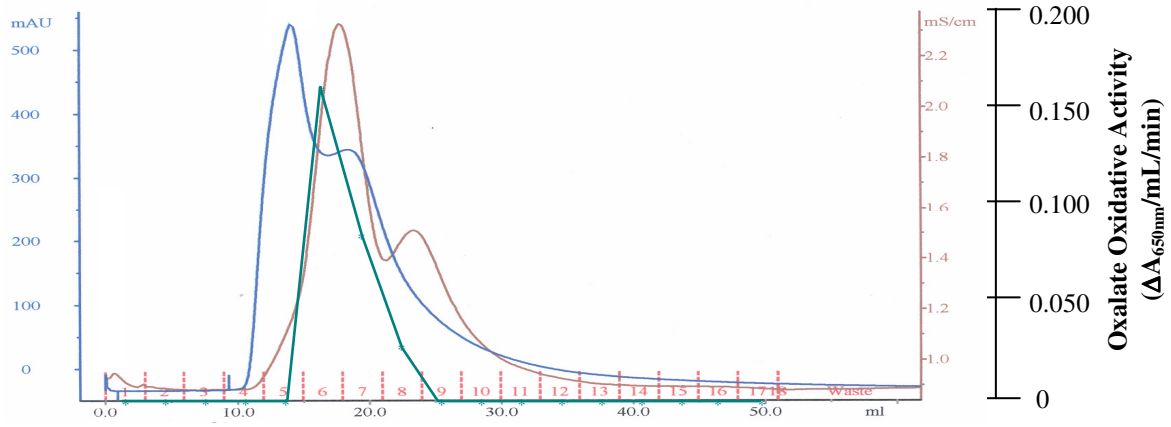
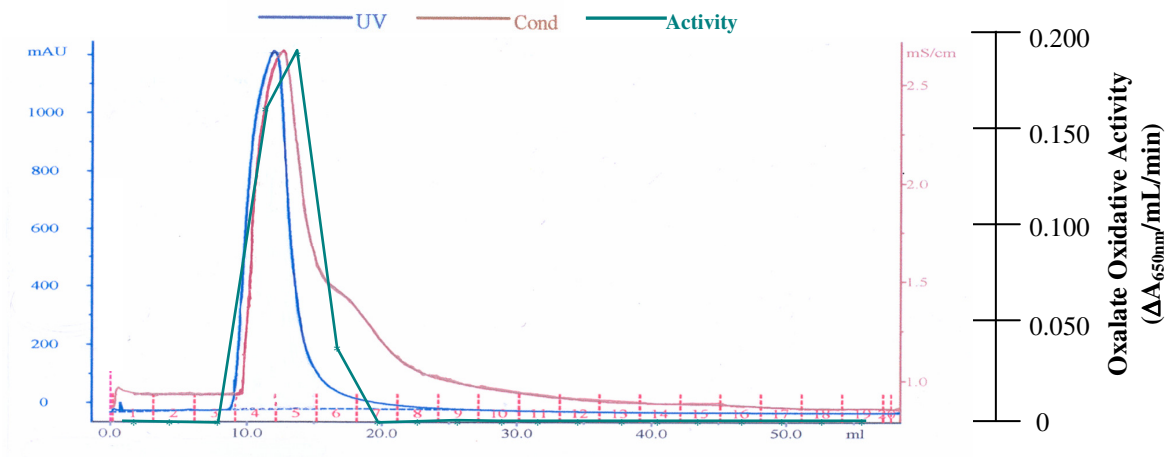


Figure 6.2: Sephadex G15 and G10 Chromatography of Cabbage Leaf Oxalate Oxidising Component.

Active cabbage leaves oxalate oxidative fractions eluted from the Sephadex G25 column were applied to the Sephadex G15 (a) and G10 (b) columns successively. Sephadex chromatography was performed following method 2.11.4 and all eluted fractions were assayed using the ABTS assay method 2.12.6 at A_{650nm} in triplicate.

(a) Sephadex G15 Chromatography



(b) Sephadex G10 Chromatography

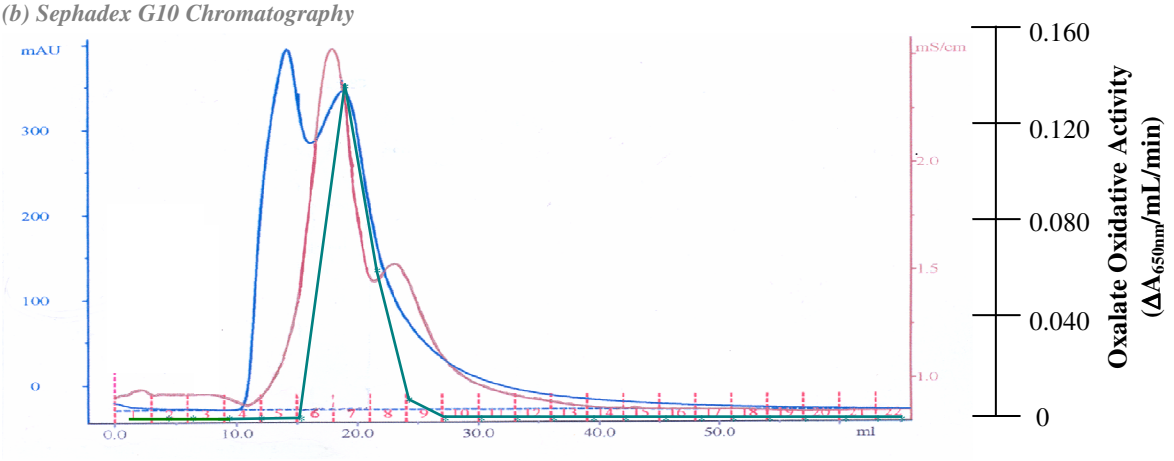


Figure 6.3: Sephadex G15 and G10 Chromatography of Mint Leaf Oxalate Oxidising Component.

Active mint leaves oxalate oxidative fractions eluted from the Sephadex G25 column were applied to the Sephadex G15 (a) and G10 (b) columns successively. Sephadex chromatography was performed following method 2.11.4 and all eluted fractions (3 mL) were assayed using the ABTS assay method 2.12.6 at A_{650nm} in triplicate.

It was noted during Sephadex G10 and G15 chromatography that the oxalate oxidising components not only eluted with the salt peak, but also eluted with the A_{280nm} absorbing material suggesting they contained aromatic or conjugated compounds. In conclusion, Sephadex G15 and G10 chromatography of the oxalate oxidising components in cabbage and mint leaves exhibited similar separational characteristics.

Since the fractionation range of Sephadex G10 media is equal to or less than 700 Da, it was concluded that the oxalate oxidising components in cabbage and mint leaves were less than

700 Da. These studies suggest carrot leaves oxalate oxidative component may also be less than 700 Da since it exhibited similar separation characteristics to cabbage and mint leaves during Sephadex G25 chromatography. To further investigate the molecular mass of the cabbage, carrot and mint oxalate oxidising components, dialysis was undertaken.

6.1.2 Dialysis of Plant Extracts

To confirm the molecular mass of the oxalate oxidising components in the three sources, the extracts were subjected to dialysis. Dialysis was performed for 24 hours at 4 °C using dialysis tubing with a molecular mass cut off of 12 kDa, following method 2.8. Molecular mass components less than 12 kDa were expected to diffuse out of the dialysis membrane pores into the surrounding buffer. To monitor the activity of the dialysed extracts, undialysed preparations were also stored at 4 °C for 24 hours, after which both extracts were assayed for oxidative activity, shown in table 6.1.

	Rate of Oxalate Oxidation (ΔA_{650nm}/min/mL)	
	Stored at 4 °C for 24 Hours	**Dialysed at 4 °C for 24 Hours**
Cabbage Leaves	0.612 ± 0.056	0.009 ± 0.002
Carrot Leaves	1.934 ± 0.187	0.039 ± 0.009
Mint Leaves	3.713 ± 0.179	0.187 ± 0.020

Table 6.1: **Comparison of the Oxalate Oxidative Activities in Dialysed and Undialysed Plant Extracts.**

Dialysis was performed with thermally treated extracts at 4 °C for 24 hours following method 2.8. Enzymatic assays were carried out using the + oxalate ABTS assay at A_{650nm} in triplicate following method 2.12.6.

Enzymatic assays performed after dialysis showed a loss of 99 %, 98 % and 95 % oxidative activity in cabbage, carrot and mint leaves respectively compared to undialysed extracts. These studies prove that the oxidising components in cabbage, carrot and mint leaves were less than 12 kDa, in agreement with the Sephacryl S-200-HR (chapter 5.9) and Sephadex chromatography data. These findings also confirm the interpretation of the data obtained in chapter 5.6 during ammonium sulphate fractionation (ASF) where most of the activity was reduced after ASF and dialysis.

To further confirm the oxalate oxidising components in cabbage, carrot and mint leaves were less than 700 Da and not associated with protein eluting fractions, where Oxox was expected, protein electrophoresis was performed.

6.1.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of Partially Purified Extracts

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed on the extracts which had been subjected to Sephadex G25 chromatography. Polyacrylamide gels were initially stained with Coomassie Blue according to method 2.15, however, the oxalate oxidising components were not visible on the gel. To overcome this, the silver nitrate staining technique was employed (method 2.16) since this stain detects up to 0.1 μg to 0.5 μg protein compared to that of the less sensitive 1 μg to 5 μg for Coomassie Blue [139].

SDS PAGE was performed, following method 2.14 on a selection of cabbage leaves fractions eluted from Sephadex G25 chromatography:

- fraction prior to the elution of the A_{280nm} absorbing material (fraction 3)
- fractions from the first major A_{280nm} absorbing peak (fractions 6 and 8)
- fractions containing the oxalate oxidising component (fractions 13 and 14)
- fractions from the second major A_{280nm} peak (fractions 17, 20, 24, 28, 32 and 36)

The fractions listed above are represented by the shaded region in figure 6.4a and the resultant polyacrylamide gel is shown in figure 6.4b after staining with silver nitrate.

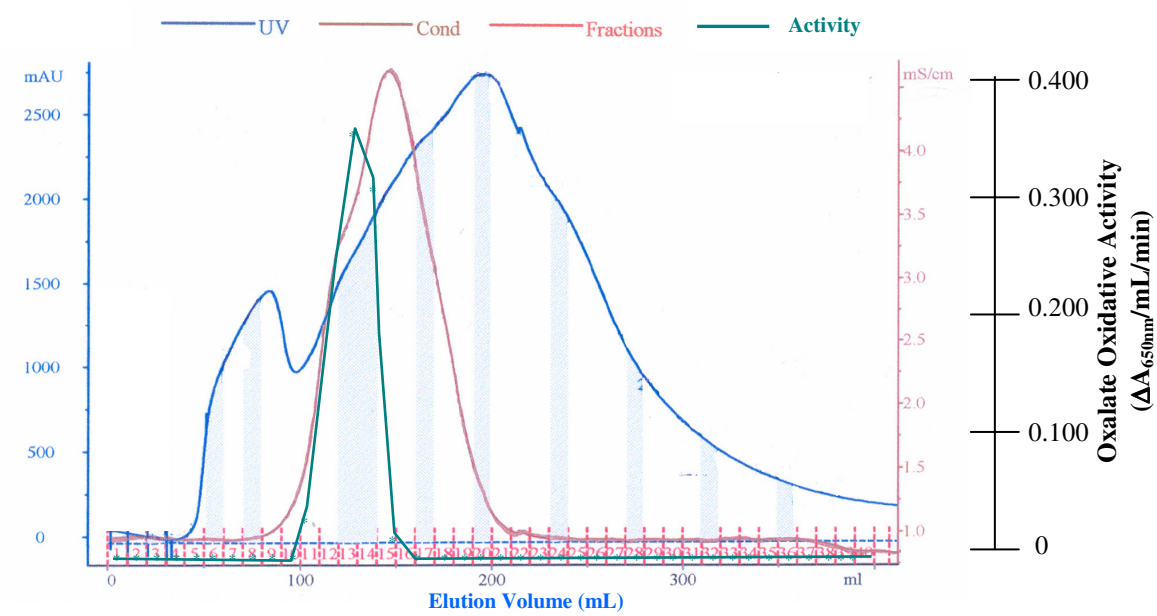


Figure 6.4a: Sephadex G25 Chromatography Elution Profile Illustrating Fractions Analysed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.

Shaded areas indicate the fractions selected for SDS PAGE analysis.

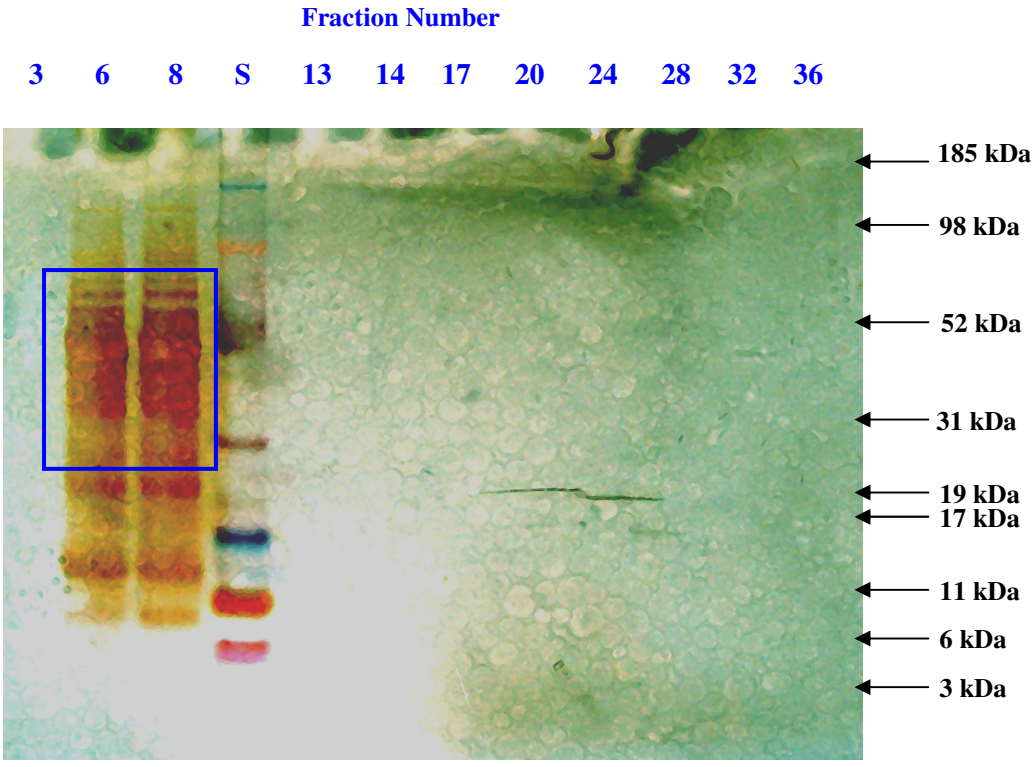


Figure 6.4b: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of Sephadex G25 Chromatography Fractions.

SDS PAGE was performed following method 2.14 and silver stained following method 2.16. The fraction numbers and molecular masses of the molecular standards (S) are indicated.

SDS PAGE resolved proteins between 3 kDa and 185 kDa, and led to the visualisation of proteins in fractions 6, 8 and the molecular standards only. It is evident from figure 6.4b that fractions 6 and 8 contain proteins between approximately 10 kDa and 150 kDa. Purified Oxox has been reported to have a molecular mass of between 25 kDa [85] to 75 kDa [44, 84] for individual subunits. If Oxox was present in the extract, it would have been visualised within the region indicated on the polyacrylamide gel in figure 6.4b.

The inability to visualise proteins in fractions 13, 14, 17, 20, 24, 28, 32 and 36 may have been due to these fractions not containing denatured proteins between 3 kDa and 185 kDa, or alternatively, the fractions analysed contained insufficient protein concentrations. The latter suggestion was eliminated after performing Lowry and Bicinchoninic acid (BCA) protein assays which demonstrated adequate protein concentrations were present. Both Lowry and BCA assays contain copper sulphate, whereby the cupric ions form a coordination complex with amide groups which are generally provided by peptide bonds linking amino acids in proteins.

SDS PAGE was also undertaken with the active carrot and mint oxidising fractions eluted during Sephadex G25 chromatography. Similar findings were obtained during these studies where the components of interest were not visible on the polyacrylamide gels.

To conclude, the chromatographic, dialysis and electrophoretic studies suggest the oxalate oxidising components in cabbage, carrot and mint leaves are not likely to be proteins, although they strongly absorb at A_{280nm}. To provide further evidence for the molecular mass of the oxidising components, further gel filtration chromatography was undertaken.

6.1.4 Bio Gel P2 Gel Filtration Chromatography of Cabbage Leaf Extract

To resolve and further identify the low molecular mass oxalate oxidising components, Bio Gel P2 chromatography was performed since this possesses a fractionation range of 100 Da to 1800 Da. Only cabbage extract was subjected to this procedure since this extract was available in larger quantities in comparison to carrot and mint leaves.

Since larger quantities of the cabbage Sephadex G25 eluted extract was available, further gel filtration chromatography was undertaken using this extract. The active cabbage

oxalate oxidative extract after Sephadex G25 was assayed using both + and – oxalate ABTS assay mixtures prior to undertaking Bio Gel P2 chromatography. A typical chromatogram obtained after the purification of the oxidising component in cabbage leaves using Bio Gel P2 chromatography according to method 2.11.5 is shown in figure 6.5.

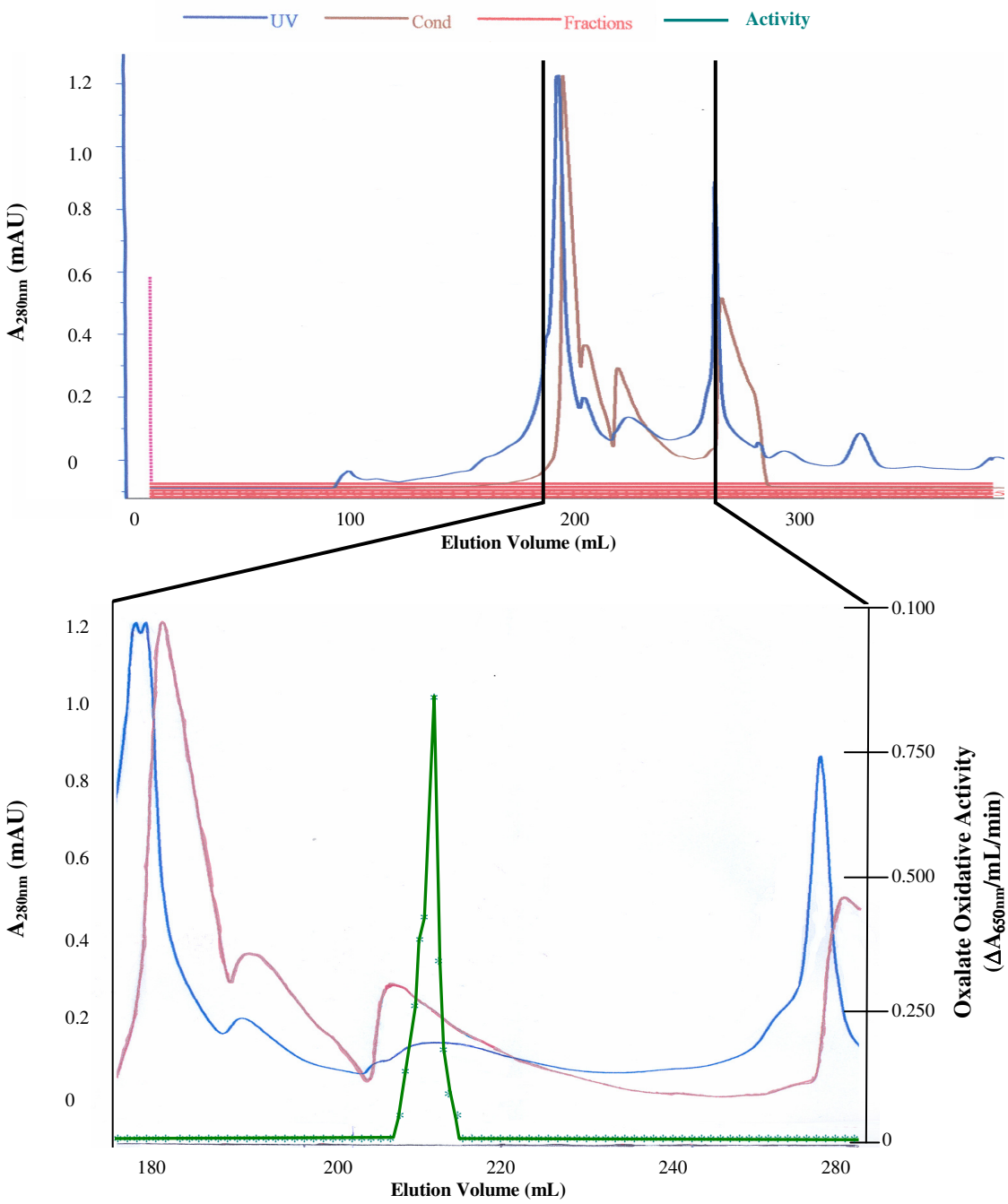


Figure 6.5: Bio Gel P2 Chromatography of the Oxalate Oxidising Component in Cabbage Leaves.

Active oxalate oxidising fractions eluted during Sephadex G25 chromatography were applied to the Bio Gel P2 column following method 2.11.5. All fractions eluted were assayed using the ABTS assay at A_{650nm} in triplicate following method 2.12.6.

Further chromatography of the active cabbage leaves oxalate oxidising component using Bio Gel P2 chromatography led to the resolution of four major A_{280nm} absorbing components greater than 0.2 mAU.

Enzymatic assays undertaken led to the elution of cabbage leaves oxalate oxidising component between 208 mL and 217 mL during Bio Gel P2 chromatography, shown in figure 6.5. This chromatographic procedure successfully resolved the oxalate oxidising component from at least four other major components.

6.1.4.1 Staining Properties of Plant Extracts

Protein electrophoresis performed in section 6.1.3 led to the inability to visualise the cabbage, carrot and mint oxalate oxidising components. During these studies the protein content of the extracts applied to the polyacrylamide gels after staining with Coomassie Blue and silver nitrate were determined using two types of protein quantification assays: Lowry and BCA.

Lowry protein assay possesses a detection limit of 1 mg of protein per mL [138], however, the BCA assay is more sensitive since it is capable of detecting protein concentrations as low as 200 µg to 1000 µg per mL [138]. Both these assays form copper-protein complexes whereby the cupric ions form a coordination complex with nucleophilic amine groups [142]. Since both of these protein assays contain copper sulphate, the copper will interact with a number of functional groups including amines, amides and carboxylic acids in any given compound, including the cabbage, carrot and mint oxalate oxidising components. The protein quantification studies undertaken during SDS PAGE generated a positive result, whereby copper in the Lowry and BCA assay components interacted with functional groups within the oxidising components. This in turn indicates the oxalate oxidising components in cabbage, carrot and mint extracts contain functional groups which formed copper complexes when analysed by Lowry and BCA assays.

6.2 UV/VIS SPECTROSCOPY OF CABBAGE AND MINT OXALATE OXIDISING COMPONENTS

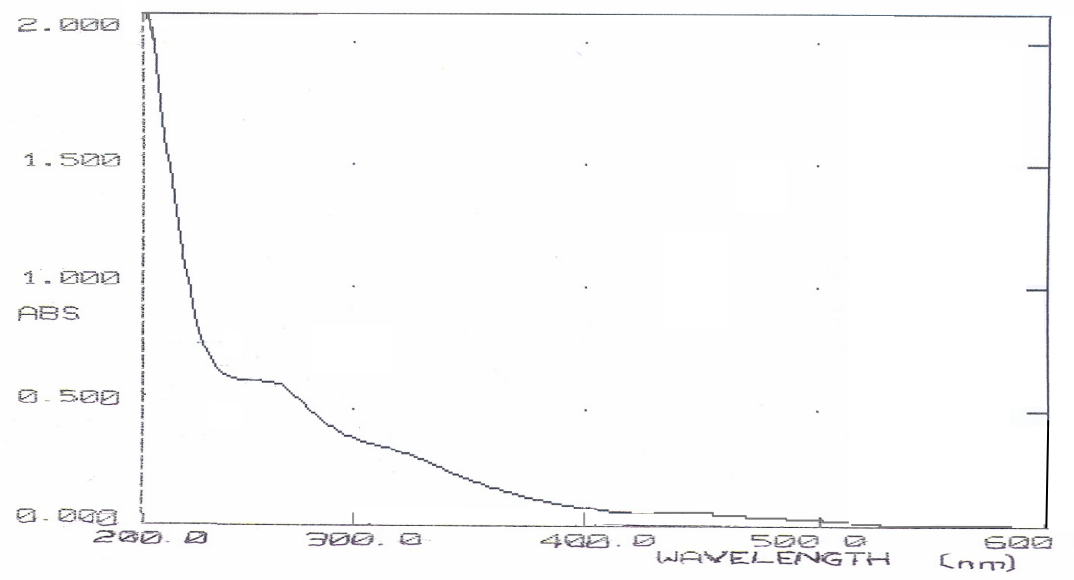
Further studies with cabbage and mint leaves were undertaken, since larger quantities were available. Spectral studies were undertaken to characterise the oxalate oxidising components in cabbage and mint leaves. The mint extract eluted during Sephadex G10 chromatography was used during these studies, however, the highly pure cabbage oxalate oxidising component eluted during Bio Gel P2 chromatography was used to determine the spectral properties of the extracts. The absorbance of cabbage and mint oxalate oxidising components were measured between 200 nm and 600 nm, the resultant spectra are shown in figure 6.6a (cabbage leaves) and 6.6b (mint leaves).

The absorption spectrum of the highly pure cabbage leaves oxalate oxidising extract (figure 6.6a) showed weak absorption at approximately 255 nm and 310 nm. Conversely, the mint leaves active component in figure 6.6b absorbed at approximately 260 nm and 320 nm.

Since chromatographic analysis undertaken during this research detected eluting components at 280 nm, both extracts exhibited absorption properties around the region of 280 nm, visible on figures 6.6a and 6.6b respectively.

The absorption spectra obtained for both the cabbage and mint oxalate oxidising components were similar, however, no certain conclusion can be drawn from these findings since the two extracts analysed were purified using different protocols and required dilution. Therefore, it cannot be concluded for certain whether both extracts contained similar or different oxalate oxidising components.

(a) Cabbage Leaves



(b) Mint Leaves

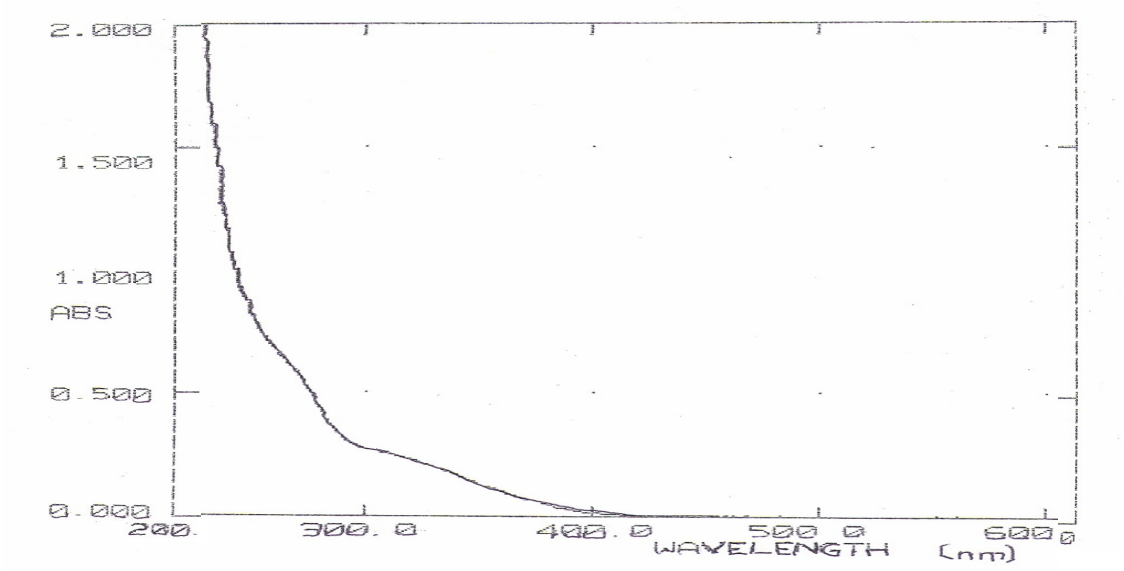


Figure 6.6: Absorption Properties of the Oxalate Oxidising Components in Cabbage and Mint Leaves.

The active cabbage oxalate oxidising component (a) eluted from the Bio Gel P2 column and the mint leaves oxidative component (b) eluted during Sephadex G10 chromatography were scanned from 200 nm to 600 nm.

During initial UV/Vis spectroscopic analysis of cabbage leaves oxalate oxidative component, the spectrum obtained differed to that of the same extract shown in figure 6.6a. The extract analysed was in the presence of tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) buffer, the resultant spectra is shown in figure 6.7.

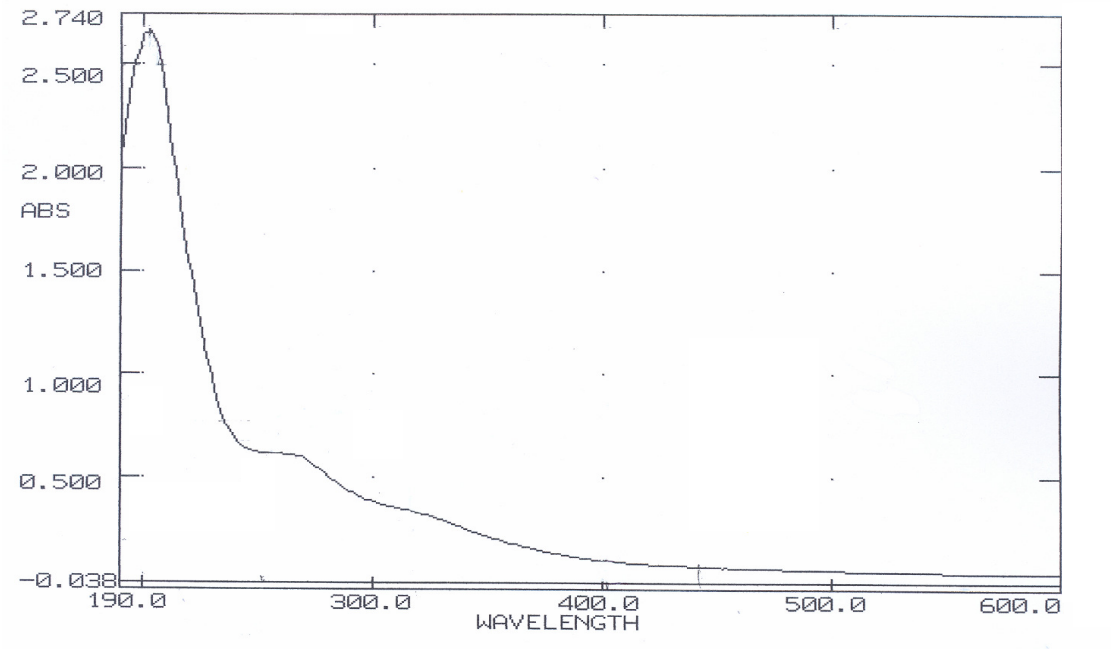


Figure 6.7: Absorption Properties of the Oxalate Oxidising Component in Cabbage Leaves.

The active cabbage oxalate oxidising component eluted from the Bio Gel P2 column, in the presence of Bis Tris were scanned from 200 nm to 600 nm.

UV/Vis spectroscopy of cabbage extract in the presence of tris buffer showed absorbance at 203 nm, 260 nm and 315 nm, however the same extract in the absence of this buffer absorbed at 255 nm and 310 nm only.

The shift in absorption to longer wavelengths was due to the presence of tris buffer which possesses one amine and three hydroxyl groups. The dissimilarities in absorption properties may be due to two potential factors. Firstly, the presence of tris may have altered the composition of the cabbage oxalate oxidative component, or secondly, the change in the pH due to the addition of tris buffer at pH 7.5 may have led to the shift in absorption peaks. It is difficult to predict the effect the amine and hydroxyl group, and the pH had on the cabbage oxalate oxidative component, however, it is evident that the presence of these functional groups altered the absorption properties of the extract analysed. In conclusion, it is apparent that the preparation of the extract in dissimilar solutions led to a substantial change in the absorption properties.

6.3 THERMAL TREATMENT OF PLANT EXTRACTS

Thermal treatment was performed to provide further proof that the oxalate oxidising components in cabbage, carrot and mint leaves were not proteins or short polypeptides, since most proteins denature at elevated temperatures and lose their activity. Thermal treatment undertaken in chapter 5.5 at 60 °C for 10 minutes demonstrated the oxalate oxidising components were thermally stable under these conditions. To further investigate the thermal stability of the active extracts eluted during Sephadex G25 chromatography, thermal treatment was prolonged for 20 minutes at 60 °C, and at 100 °C for 60 minutes following method 2.6.1. Extracts were also stored at room temperature (approximately 22 °C), after which, both the thermally treated and untreated extracts were centrifuged and assayed to identify the loss, or alternatively, the retention of activity. The resultant oxalate oxidative activities measured after thermal treatment were compared with that of the extracts stored at room temperature for the same duration, shown in table 6.2.

		Rate of Oxalate Oxidation (ΔA_{650nm}/min/mL)		**Activity Remaining after Treatment (%)**
		Untreated	**Treated**	
Cabbage	**60 °C for 20 minutes**	1.997 ± 0.203	1.979 ± 0.177	99
Leaves	**100 °C for 60 minutes**		1.917 ± 0.211	96
Carrot	**60 °C for 20 minutes**	3.745 ± 0.199	3.723 ± 0.171	99
Leaves	**100 °C for 60 minutes**		3.708 ± 0.182	99
Mint	**60 °C for 20 minutes**	0.654 ± 0.043	0.644 ± 0.040	98
Leaves	**100 °C for 60 minutes**		0.615 ± 0.039	94

Table 6.2: Thermal Treatment of Cabbage, Carrot and Mint Leaves.

Thermal treatment of the active oxalate oxidising cabbage, carrot and mint leaves extracts collected during Sephadex G25 chromatography was carried out at 60 °C for 20 minutes and 100 °C for 60 minutes following method 2.6.1. Enzymatic assays were performed using the ABTS assay at A_{650nm} in triplicate following method 2.12.6.

Thermally treating the extracts for 20 minutes at 60 °C and at 100 °C for 60 minutes did not significantly affect the oxalate oxidative activities in the three sources. Approximately 96 % (cabbage leaves), 99 % (carrot leaves) and 94 % (mint leaves) of the activity was retained after extensive thermal treatment at 100 °C for 60 minutes.

The thermal stability of the components of interest exceeds that of most thermophilic bacteria, therefore, these studies strongly suggest the oxalate oxidising components in cabbage, carrot and mint leaves are not proteins or polypeptides since they were found to be extremely thermally stable.

6.4 OVERVIEW

Cabbage, carrot and mint oxalate oxidising components were found to be smaller than 700 Da, extremely thermally stable at 100 °C for 60 minutes and were not visualised on polyacrylamide gels after staining with either Coomassie Blue or silver nitrate. On the other hand, documented Oxox has a molecular mass between 125 kDa [86] and 400 kDa [75], stable up to 60 °C for 10 minutes [78, 85] and is clearly visible on polyacrylamide gels after staining with Coomassie Blue [70, 74, 80, 82, 86] and silver nitrate [75, 78, 85].

Lowry and BCA protein assays undertaken led to positive results whereby the copper present in the protein assays formed complexes with the functional groups in the extracts. Therefore, suggesting cabbage, carrot and mint oxalate oxidising components can act as a ligand for copper complexes.

The oxalate oxidative components in the three sources absorbed at A_{280nm} during Sephadex and Bio Gel P2 chromatography. These findings demonstrated the components of interest contained aromatic compounds, or alternatively, a conjugated organic system. UV/Vis spectroscopy undertaken with cabbage and mint leaves confirmed the absorption properties of these components in the region around 280 nm.

Cabbage, carrot and mint oxalate oxidising components were found to be extremely thermally stable for 60 minutes at 100 °C, proving the components of interest were not proteins or short polypeptides, however were more likely to be organic molecules.

The isolation and partial purification of the oxalate oxidising components from the three sources demonstrates the components isolated may be novel oxalate oxidative conjugated compounds. Further analysis of the cabbage and mint oxalate oxidising components was therefore undertaken to characterise and to identify if the oxidative components were

cofactors which may have dissociated from their host proteins during purification procedures.

6.5 COFACTOR

It was suggested the oxidising components in cabbage and mint leaves components of interest may be cofactors which had dissociated from their host proteins during chromatographic procedures. Cofactors can either be metal ions such as Cu^{2+}, Fe^{3+} or Mn^{2+}, or alternatively organic molecules such as NAD^{+}. Some cofactors are tightly bound to their host protein, whereas others can be easily dissociated [143].

Oxox (E.C. 1.2.3.4) is a member of oxidoreductase (E.C. 1.) enzymes and utilises oxygen as an accepter, yielding hydrogen peroxide and carbon dioxide during the reaction. Oxox is part of a subclass of enzymes which act on the aldehyde or Oxo group of donors (E.C. 1.2). This group can be divided into further subclasses which utilise the following cofactors during a reaction:

- E.C. 1.2.1 - NAD^{+} or $NADP^{+}$
- E.C. 1.2.2 - Cytochrome
- E.C. 1.2.3 - Oxygen
- E.C. 1.2.4 - Disulphide
- E.C. 1.2.7 - Iron-sulphur
- E.C. 1.2.99 – Other

Most cofactors utilised during oxidoreductase reactions are only functional in conjunction with an enzyme, with the exception of E.C. 1.2.99 cofactors. Therefore, the likelihood of the oxalate oxidising component being NAD^{+}, Cytochrome, oxygen, disulphide or iron-sulphur cofactors was disregarded since these cofactors have not been known to function independently.

It was suggested that the isolated cabbage, carrot and mint oxidising components may be acting as acceptors during the enzymatic reactions performed during this research. There are a number of ‘unknown’ E.C. 1.2.99 cofactors reported which are utilised during oxidase

reactions, the only known cofactor, pyrroloquinoline quinone (PQQ) can function independently without an enzyme. PQQ is relatively small cofactor with a molecular mass of 330 Da and is shown in figure 6.8.

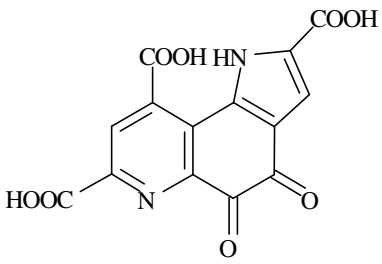


Figure 6.8: Structure of Pyrroloquinoline Quinone.

PQQ is known to function independently from its host protein and is purified using protocols such as ion exchange chromatography. This procedure employs a salt gradient to remove PQQ from its host protein, therefore, the possibility of the oxalate oxidative components in the three sources being PQQ was a viable suggestion since the active components eluted with the salt components during Sephadex chromatography. Additionally, the molecular mass, presence of amine and acid groups, and conjugation properties of PQQ coincide with the characteristics of cabbage, carrot and mint oxalate oxidising components.

PQQ contains an amine group, as do the oxalate oxidative components in cabbage, carrot and mint leaves, which was determined during protein assays. PQQ was analysed using BCA protein assay to determine whether it would also form similar copper-amine complexes to the oxalate oxidative components. These studies led to a positive result, whereby the neuclophilic amine reacted with the copper ions in the BCA assay, measured at 562nm forming a copper-protein complex.

The UV/Vis spectrum of PQQ was measured to compare its absorption properties to that of cabbage and mint oxalate oxidising components. PQQ was prepared in methanol since it was not soluble in water, the resultant spectral scan is shown in figure 6.9.

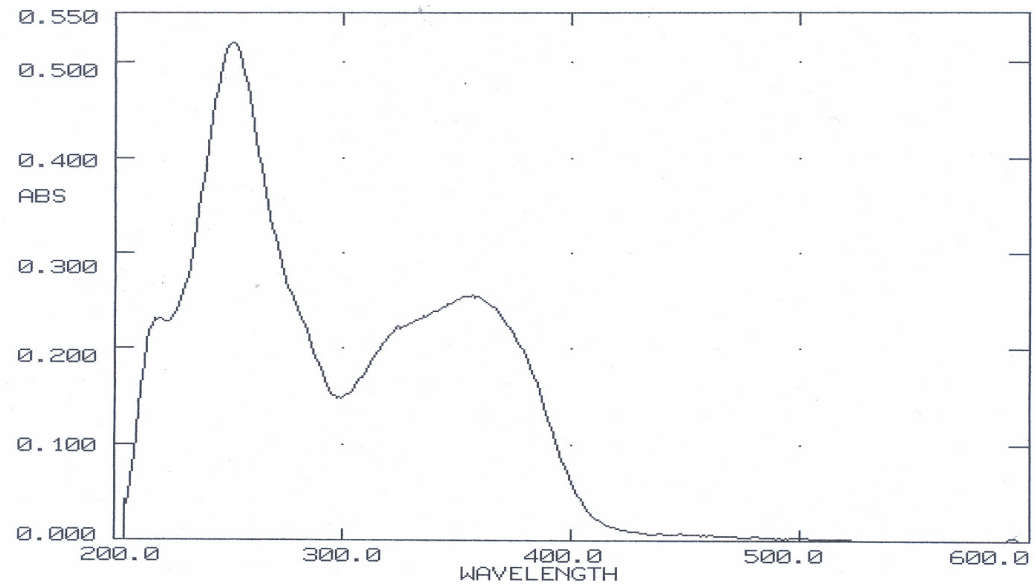


Figure 6.9: Absorption Properties of Pyrroloquinoline Quinone.

Commercially available (Sigma Aldrich Ltd.) 7.7 µg/mL PQQ prepared in methanol : water (50:50 v/v) was scanned from 200 nm to 600 nm.

The spectrum of PQQ showed absorptions at 217 nm, 252 nm, 323 nm and 357 nm, shown in figure 6.9. Since PQQ is an aromatic conjugated molecule, it was expected to absorb at 280 nm, similar to the oxalate oxidative components in cabbage and mint leaves. Comparison of the absorption properties of cabbage and mint oxalate oxidising components to that of PQQ is shown in table 6.3.

	Absorption Properties (nm)			
PQQ	217	252	323	357
Cabbage		255	310	
Mint		260	320	

Table 6.3: The Absorption Properties of Pyrroloquinoline Quinone, Cabbage and Mint Leaves Oxalate Oxidising Components.

The cabbage and mint oxalate oxidative extracts exhibited similar absorptions to PQQ at 320 nm. Since both the spectra of cabbage and mint oxalate oxidative components in figure 6.6 were not rescaled, it cannot be concluded for certain whether they possess absorption properties at 217 nm. Neither extracts analysed possessed similar absorption properties to PQQ at 217nm, 252 nm and 357 nm.

Due to dissolution problems of PQQ in water, this cofactor was prepared in methanol. The presence of methanol may have affected the absorption properties of PQQ since it is known to form adducts with methanol. The absence of a peak at 280 nm in PQQ may be due to the sample preparation, or alternatively, the spectrum may have undergone a bathochromic shift.

A bathochromic shift in an absorption spectra occurs when the extent of conjugation in a double bonded system such as PQQ is increased causing a shift in the spectral band position to a longer wavelength. In the presence of conjugated double bonds, the electronic energy levels of a chromophore move closer together, causing the wavelength of the light absorbed to become longer. It is difficult to predict the length of the chain of the conjugated PQQ analysed, the longer the conjugated chain, the greater the bathochromic shift of PQQ. The absorption properties of PQQ at 323 nm and 357 nm may have occurred due to the bathochromic shift which could have changed the spectral position of the expected 280 nm peak to 323 nm or 357 nm.

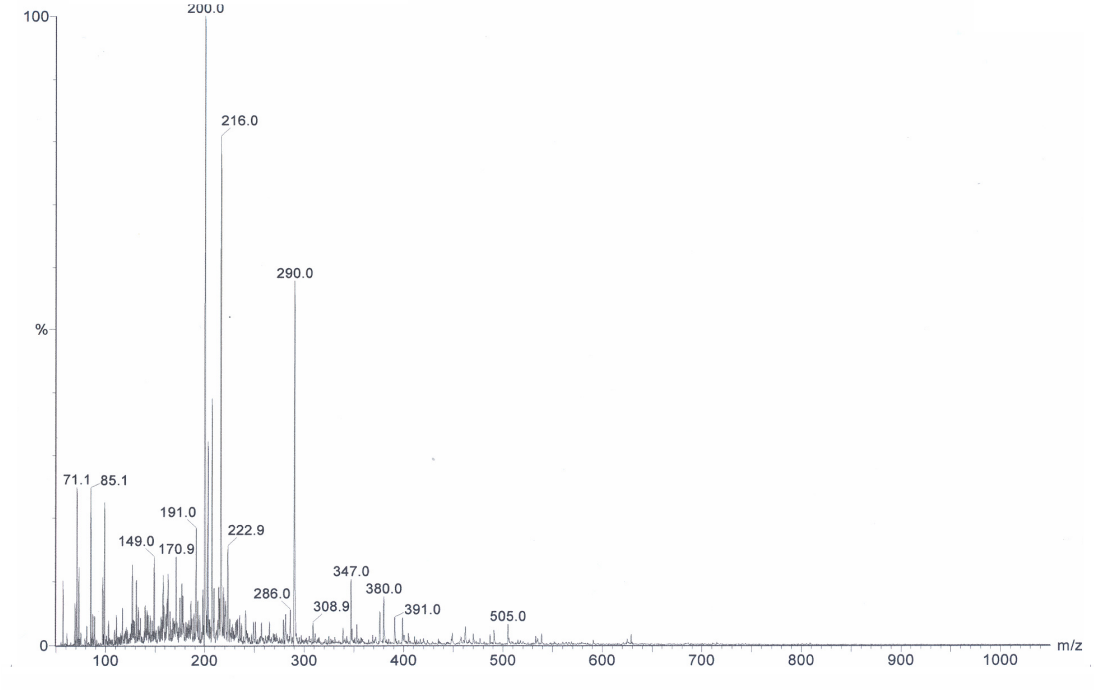
It could not be concluded for certain that the oxalate oxidising component in mint leaves were PQQ based on the molecular mass, BCA assay and absorption properties, therefore, the oxalate oxidative components in cabbage and mint leaves were characterised further to identify the component of interest.

6.6 CHARACTERISATION OF CABBAGE AND MINT LEAF OXALATE OXIDISING COMPONENTS BY MASS SPECTROMETRY

To identify the nature of the cabbage and mint leaves oxalate oxidising components, electrospray ionization mass spectrometry (ESI MS) analysis was undertaken. The analysis of the oxidising component by this technique was performed to determine the molecular mass and possible composition of the components of interest.

Positive-mode ESI MS was carried out according to method 2.17, the resultant spectrum for cabbage and mint leaves are shown in figure 6.10a and 6.10b respectively.

(a) Cabbage Leaves



(b) Mint Leaves

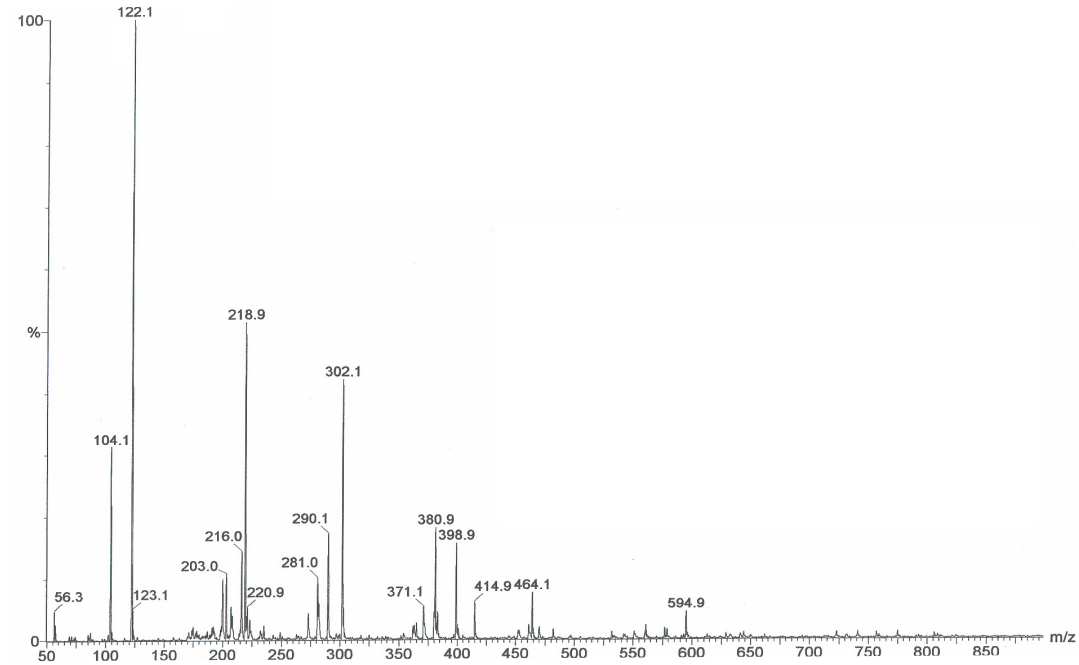


Figure 6.10: Positive-Mode Electrospray Mass Spectrometry Spectra of Cabbage and Mint Leaf Oxalate Oxidising Components.

The active cabbage leaves (a) oxalate oxidising component eluted from the Bio Gel P2 column, and the active mint leaves (b) oxalate oxidising component eluted from the Sephadex G10 column were analysed by positive-mode ESI MS following method 2.17.

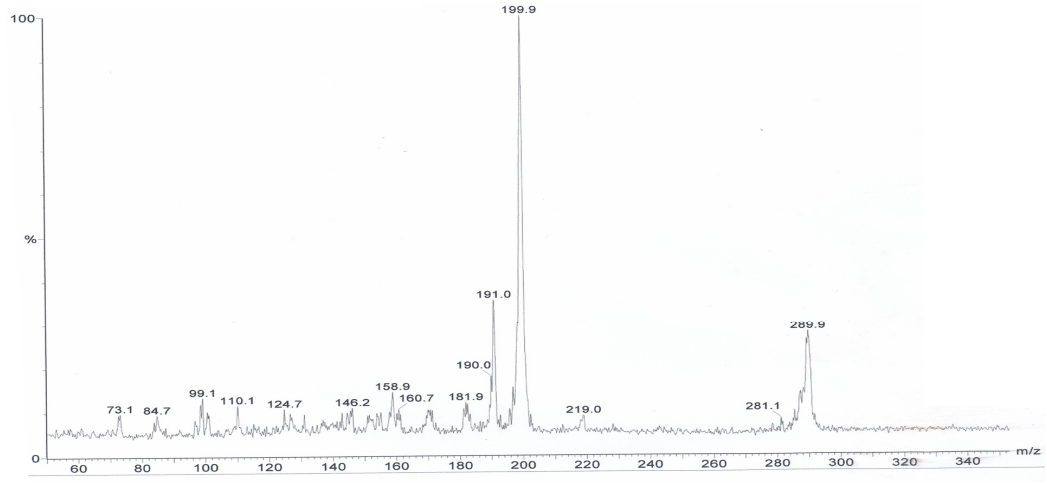
Positive-mode ESI MS led to the detection of multiple fragments in both the cabbage (figure 6.10a) and mint (figure 6.10b) leaves oxalate oxidising components. The mint extract spectrum contained additional fragments in comparison to the cabbage leaves oxalate oxidising component, suggesting both extracts possibly contained a number of components.

The spectra in figure 6.10 showed significant differences, reflecting the variation in the purity and preparation. As the cabbage extract analysed was purer than the mint extract, the cabbage preparation was selected for further positive-mode ESI MS analysis.

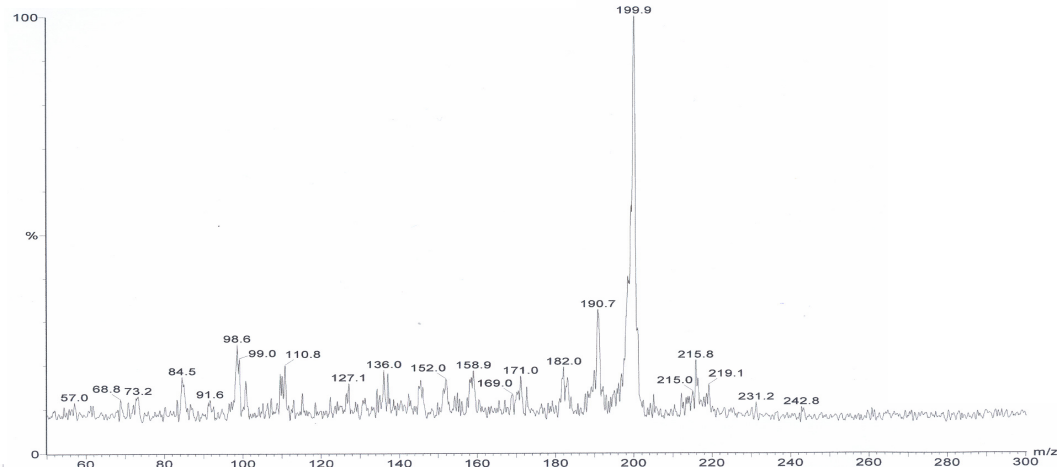
In addition to performing positive-mode ESI MS on the active oxalate oxidative fractions collected during purification, the inactive fractions were also analysed using the same technique. Three significant fragments were visualised in the active fractions, shown in figure 6.9a at masses of 200 m/z, 216 m/z and 290 m/z, however these fragments were absent in the inactive samples analysed. Further fragmentation analysis of these fragments in the cabbage oxalate oxidative extract was undertaken by performing product ion scans, shown in figure 6.11. These additional scans would determine the product ions resulting from the 200 m/z, 216 m/z and 290 m/z precursor ions.

Product ion scans of the 200 m/z, 216 m/z and 290 m/z fragments led to the finding that they were related since the 200 m/z fragment was visualised in the 216 m/z and 290 m/z fragments.

(a) Product Ion Scan of 290 m/z fragment



(b) Product Ion Scan of 216 m/z fragment



(c) Product Ion Scan of 200 m/z fragment

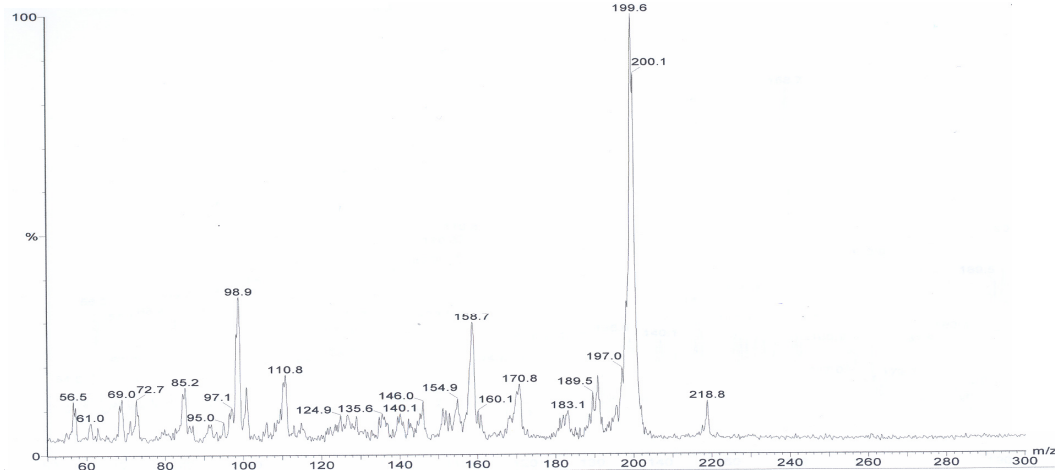


Figure 6.11: Product Ion Scans of Positive-Mode Electrospray Mass Spectrometry of the Oxalate Oxidising Component in Cabbage Leaves.

To determine if the cabbage and mint oxalate oxidising components were potentially PQQ, this cofactor was also analysed using positive-mode ESI MS. The resultant spectrum obtained for PQQ is shown in figure 6.12.

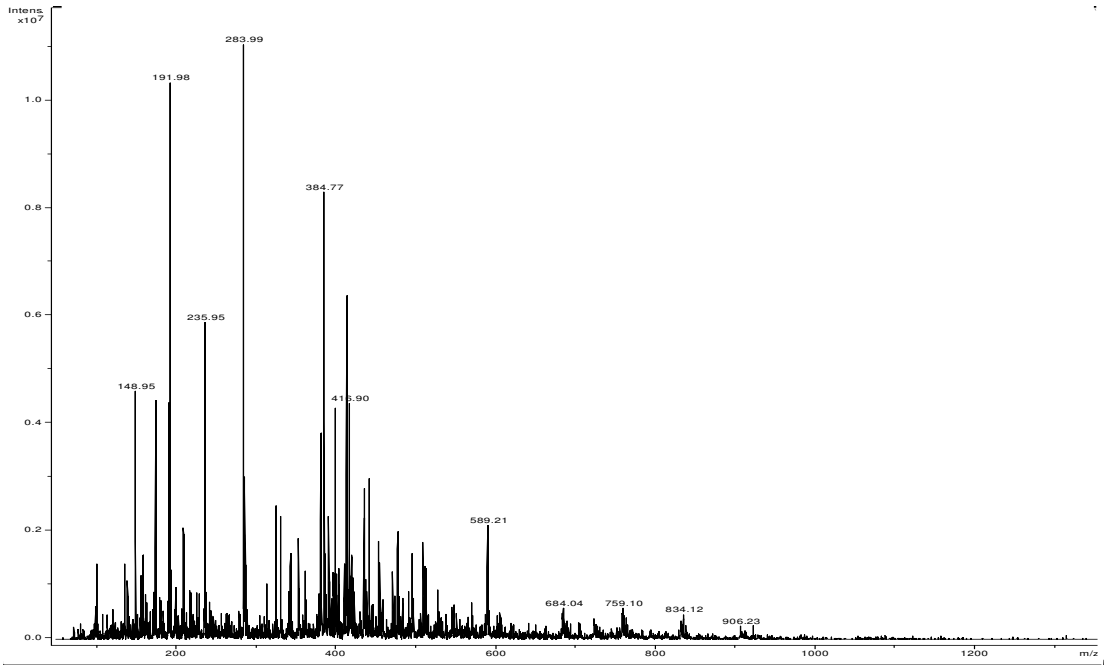


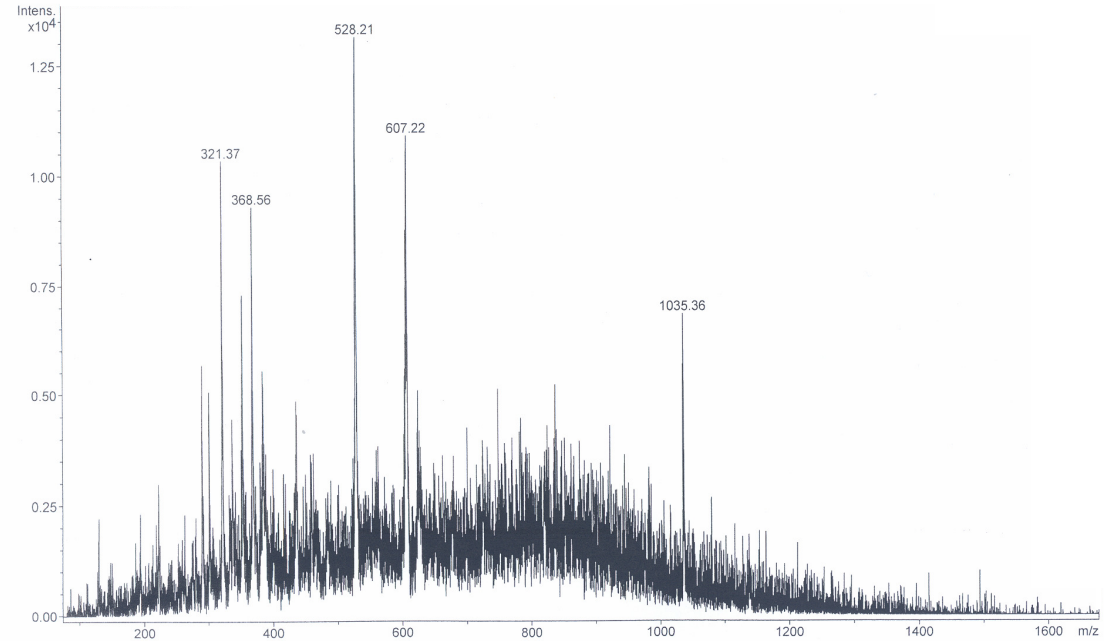
Figure 6.12: Positive-Mode Electrospray Mass Spectrometry Spectrum of Pyrroloquinoline Quinone.

Commercially available 0.1 mg/mL PQQ (Sigma Aldrich Ltd.) prepared in methanol : water (50:50 v/v) was analysed by positive-mode ESI MS following method 2.17.

The PQQ spectrum above contained a number of fragments, however, this spectrum was not comparable with the cabbage and mint oxalate oxidative components spectra since PQQ and plant extracts were prepared in dissimilar solutions. Considering 98 % HPLC grade PQQ was analysed, the resultant positive-mode ESI MS spectrum was not expected to be 'noisy'. PQQ may have formed adducts with methanol, as a result, it is difficult to determine the ions formed.

As a result, cabbage and mint oxalate oxidative components, and PQQ were analysed using negative-mode ESI MS in an attempt to clarify the spectra. The resultant spectra obtained after undertaking negative-mode ESI MS with PQQ and the plant extracts are shown in figures 6.13 and 6.14 respectively.

(a) Cabbage Leaves



(b) Mint Leaves

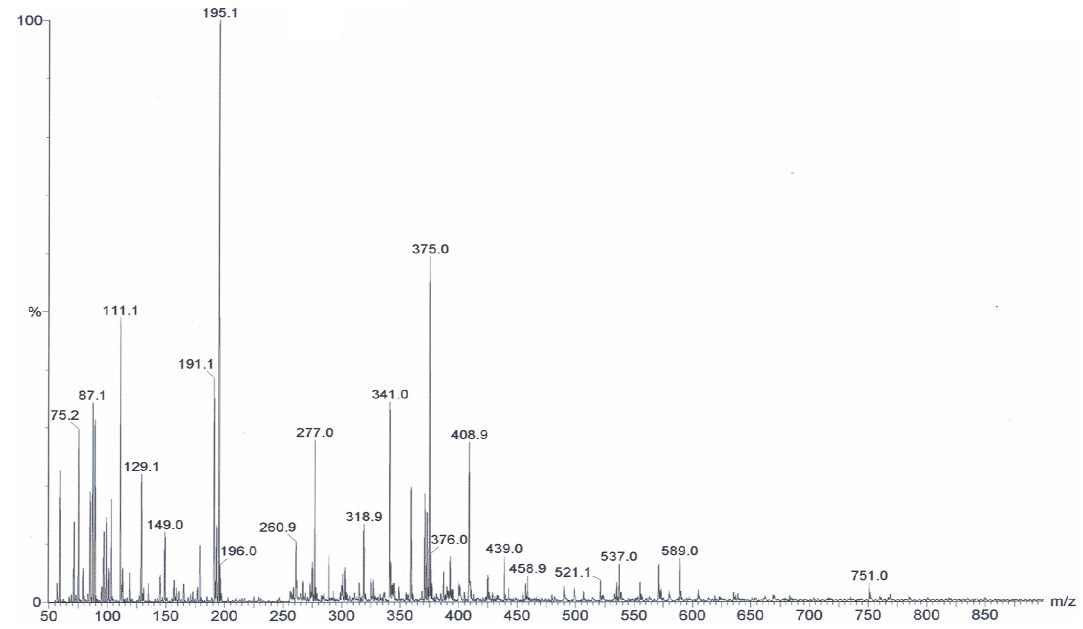


Figure 6.13: Negative-Mode Electrospray Mass Spectrometry Spectra of Cabbage and Mint Leaf Oxalate Oxidising Components.

The +active cabbage leaves (a) oxalate oxidising component eluted from the Bio Gel P2 column, and the active mint leaves (b) oxalate oxidising component eluted from the Sephadex G10 column were analysed by negative-mode ESI MS following method 2.17.

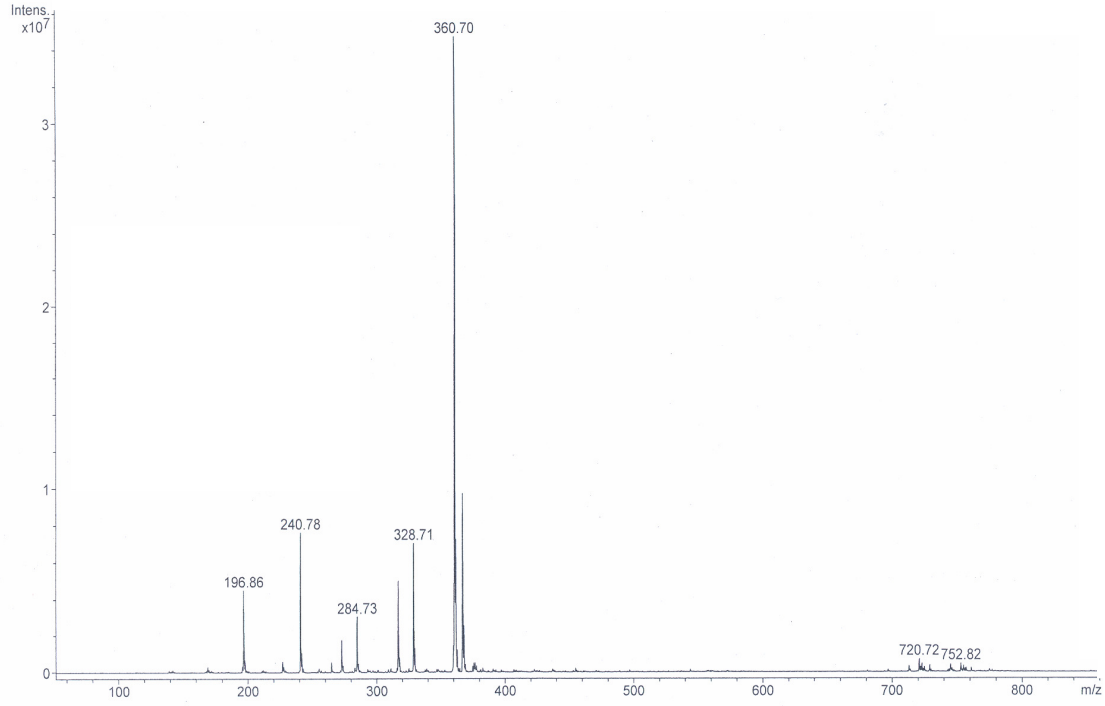


Figure 6.14: Negative-Mode Electrospray Mass Spectrometry Spectrum of Pyrroloquinoline Quinone.

Commercially available 0.1 mg/mL PQQ (Sigma Aldrich Ltd.), prepared in methanol : water (50:50 v/v) was analysed by negative-mode ESI MS following method 2.17.

Negative-mode ESI MS performed with cabbage leaves (figure 6.13a) led to the visualisation of fewer fragments in comparisons to mint leaves (figure 6.13b). The dissimilarities in fragmentation may be due to the purity of these extracts. Clear fragmentation similarities in the cabbage and mint spectra were not apparent, however, it can be concluded that both plant extracts were not the same and possibly contained a number of components. Conversely, the resultant spectrum of PQQ could be interpreted.

Negative-mode ESI MS indicated that PQQ ionized as a mixture of PQQ with $[M-H]^-$ ions at 329 m/z. It is known that PQQ readily forms adducts with nucleophilic reagents such as methanol, aldehydes, ketones, urea, ammonia and amines [144-146]. Therefore, PQQ and methanol gave $[M-H]^-$ ions at 361 m/z, the formation of this methyl hemiketal accounted for the additional 32 Da. Fragmentation of PQQ also resulted in the production of 285 m/z, 241 m/z and 197 m/z ions, showing the loss of all carboxyl groups.

The PQQ spectrum (figure 6.14) was compared to the spectra obtained with the cabbage and mint oxalate oxidative components (figure 6.13), however, no distinct similarities in fragmentation were visible. This may be due to either the plant extracts required further purification, or, the extracts were not PQQ, therefore were not comparable with PQQ.

Further analysis of the cabbage leaves active fraction was also carried out by means of ^{1}H nuclear magnetic resonance (NMR), two dimensional (^{1}H-^{1}H) correlated spectrometry (COSY) and phosphorous (^{31}P) NMR analysis (results not shown). The analysis of the oxalate oxidative components in cabbage leaves using these techniques was not very informative. However, the ^{31}P NMR undertaken demonstrated the active cabbage leaves component did not contain phosphorus groups, confirming the finding that the oxidising component was not $NADP^{+}$ like cofactors.

6.7 CONCLUSION

The molecular mass of the oxalate oxidising components in the three sources were determined by performing dialysis, Sephadex G25, G15 and G10, and Bio Gel P2 chromatography. The molecular mass of the plant extracts was determined to be less than 700 Da, demonstrating the dissimilarities in molecular mass of these oxidising components and documented Oxox. The components were found to absorb at 280 nm throughout chromatographic studies, suggesting the components contained aromatic properties. The absorption properties of cabbage and mint oxalate oxidative components were determined by UV/Vis spectroscopy, this confirmed the absorption of the plant extracts in the region of 280 nm. Initial UV/Vis analysis conducted with cabbage oxalate oxidative component in the presence of tris buffer led to a dissimilar absorption spectra. This may be due to two factors, either the pH of the tris buffer, or the functional groups in the buffer may have caused the absorption peaks to shift to a longer wavelength. These studies highlighted the impact of altering the conditions of the oxalate oxidative component.

Gel electrophoresis confirmed that no proteins were present in the active fractions. However protein quantification assays, Lowry and BCA, both generated a positive result, whereby the copper in the assays formed complexes with the functional groups in cabbage, carrot and mint oxalate oxidative components.

Since the components of interest were found to contain aromatic or conjugated organic components and were smaller than 700 Da, further studies were undertaken to determine whether the components of interest were proteins or polypeeptides, by means of extensive thermal treatment. The extracts were found to be extremely thermally stable at 100 °C for 60 minutes, illustrating the oxalate oxidising components in cabbage, carrot and mint leaves were indeed neither proteins nor polypeptides. Therefore, it was suggested that the oxalate oxidative components were possibly conjugated organic molecules rather than aromatic compounds, which would be expected in proteins.

It was proposed the oxidising components in cabbage, carrot and mint leaves may be cofactors which had dissociated from their host proteins during chromatographic procedures. This suggestion led to the evaluation of cofactors which act as acceptors in aldehyde and Oxo reactions (E.C. 1.2), one enzyme of which is Oxox. The group E.C. 1.2.99 utilise 'other' cofactors, many of which are unknown, however, a known 'other' cofactor, PQQ was researched.

PQQ can function independently from its host protein and is isolated using ion exchange chromatography using a salt gradient. It was proposed the oxalate oxidative components may be this cofactor since they produced a positive result with the BCA acid, and eluted with salt components during Sephadex chromatography, which may be mimicking a salt gradient.

Protein quantification studies with PQQ were undertaken using BCA protein assay, these led to positive results, coinciding with the finding obtained with the oxalate oxidative components. PQQ was also analysed using UV/Vis spectroscopy, and led to the finding that it absorbed at 217 nm, 252 nm, 323 nm and 357 nm. Since PQQ is a conjugated molecule, it was proposed a bathochromic shift may have occurred, changing the position of the expected 280 nm absorption peak to a longer wavelength.

To characterise PQQ, and cabbage and mint oxalate oxidative components, ESI MS was undertaken. Positive-mode ESI MS led to significant dissimilarities in the fragmentation of the plant extracts. Conclusive findings could not be made from these results, since the samples analysed differed in purity. The analysis of PQQ using negative-mode ESI MS

was successful since the resultant fragments could be identified. Conversely, negative-mode ESI MS spectra of cabbage and mint leaves were unrelated. This suggested the plant extracts were dissimilar, furthermore, were not PQQ.

Further analysis was undertaken to identify the oxalate oxidative component in cabbage leaves. A number of analyses were performed with the oxalate oxidative component in cabbage leaves including ^{1}H NMR, two dimensional COSY and ^{31}P NMR studies. These analytical studies led to the finding that cabbage leaves do not possess phosphorus groups, however, the oxalate oxidising component in cabbage and mint leaves could not be identified.

CHAPTER 7

CONCLUSION

This chapter summaries the research undertaken and highlights the overall objectives and results obtained. The original aim in chapter 1.3 was to purify an alternative source of oxalate oxidase (Oxox) for use on a biosensor system to detect oxalate in patients suffering from primary hyperoxaluria (PH). Prior to the purification of Oxox, the properties of commercially available Sigma Aldrich Ltd. Oxox were investigated.

7.1 COMMERCIAL OXALATE OXIDASE

An optimum enzymatic assay for Sigma Oxox was determined using the ABTS, aminophenazone and MBTH assays. Assays performed with the MBTH assay led to the detection of elevated Oxox activity in comparison to the ABTS and aminophenazone assays. As a result, the MBTH assay was used to study Sigma Oxox since it was found to be more sensitive.

The substrate specificity of Sigma Oxox was investigated using two sets of potential substrates, one of which were novel, the other had been investigated by various research groups. The substrates selected either possessed a di-acid group, or contained a different functional group. Neither set of potential substrates were oxidised by Oxox, demonstrating the specificity of Sigma Oxox for oxalate.

Optimum buffer studies were undertaken using 45 mM sodium succinate pH 3.8, 50 mM sodium phosphate pH 7.0 and 10 mM sodium acetate pH 5.0, whereby Sigma Oxox was prepared in these buffers and assayed. The assays conducted led to the measurement of elevated Oxox activities after preparing the enzyme in 45 mM sodium succinate pH 3.8 in comparison to those generated after preparing Oxox in the latter two buffers. Having established an optimum buffer, further studies were undertaken to determine an optimum pH for this buffer. The pH profile constructed illustrated pH 3.8 was in fact the optimum pH for 45 mM succinate, deviations in this pH led to depleted Oxox activity.

Once the substrate specificity, optimum buffer and optimum pH for Sigma Oxox were established, the purification of the commercial enzyme using cation exchange chromatography and affinity chromatography was initiated. The purification of Sigma Oxox was undertaken to achieve two objectives, firstly to establish purification protocols, and secondly to standardise chromatographic procedures which would be employed to

purify Oxox from alternative sources. The initial purification procedure performed, cation exchange chromatography was partially successful with the elution of Oxox at the reported concentration of sodium chloride (0.1 M). This procedure was problematic in the sense that inappropriate percentage yields were obtained (32.4 % ± 3.9), as a result, the stability of Sigma Oxox was investigated. Sigma Oxox was found to be unstable in sodium chloride, potassium chloride, magnesium chloride, sodium sulphate and manganese chloride, demonstrating the enzyme was Na^+ and Cl^- sensitive. As a result, the purification of Sigma Oxox using cation exchange chromatography was not pursued further.

To continue with the purification of Sigma Oxox, affinity chromatography was employed. The purification of the enzyme using this procedure led to inappropriate percentage yields (67.3 % ± 5.9), posing questions regarding the solubility of Sigma Oxox. To investigate this, dissolution studies were undertaken and led to the finding that Sigma Oxox was insoluble since it did not dissolve after vortex mixing, dialysis, sonicating and constant stirring. Since Sigma Oxox was found to be unstable and insoluble, the further purification of this enzyme was ceased.

The commercial enzyme was amperometrically analysed to determine the repeatability and reproducibility to detect oxalate. The linear range of the Sigma Oxox sensor exceeded that of many research groups [31, 35, 140] demonstrating the successfulness of the sensor developed. The data generated during amperometric studies was accurate and reliable, demonstrating the reusability and reproducibility of the Sigma Oxox sensor.

7.2 OXALATE OXIDASE FROM BARLEY ROOTS

Since the commercial enzyme is purified from barley roots, the purification of Oxox from this source was undertaken to establish working purification procedures. Barley roots Oxox isolated from the soluble component of the root and partially purified using a five step purification protocol employing subcellular fractionation, thermal treatment, ammonium sulphate fractionation (ASF), cation exchange chromatography and gel filtration chromatography.

Enzymatic assays performed with barley roots after subcellular fractionation led to the measurement of ‘oxalate oxidase’ activity in addition to ‘oxidase’ activity after assaying

using + and – oxalate assay mixtures respectively. The oxidase activity generated using – oxalate assay mixture was unconventional, however, this reduced to almost undetectable levels during the purification of barley root Oxox.

Oxox in barley roots was found to be thermally stable up to 60 °C for 10 minutes and was successfully concentrated using 30-65 % ASF. The concentrated barley root extract was purified using cation exchange chromatography, whereby Oxox eluted with approximately 0.1 M sodium chloride.

Two barley root Oxox extracts were purified using gel filtration chromatography: Oxox active samples after ASF, and after cation exchange chromatography. The purification of these extracts led to the elution of Oxox using similar volumes. However, gel filtration chromatography of the latter extract led to the enhanced resolution of distinctive peaks in comparison to the ASF extract. The resultant Oxox activity measured was substantially lowered, therefore, barley root Oxox was not purified further.

The purified enzyme was analysed amperometrically to determine its ability to detect sequential additions of oxalate. Extracts after each purification procedure were immobilised onto a platinum electrode and led to enhanced and more sensitive detection of oxalate whilst the extract was purified further, illustrating the requirement for purification. The linear range of the sensor developed exceeded that of the Sigma Oxox sensor, demonstrating the successful development of the barley root Oxox biosensor.

7.3 ALTERNATIVE SOURCES OF OXALATE OXIDASE

Having established a working purification protocol, the isolation of an alternative source of Oxox was initiated. A number of families: *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Lamiaceae*, *Fabaceae, Poaceae* and *Solanaceae* families were screened to detect the presence of Oxox activity. A number of potential sources of Oxox were determined, however some were disregarded after performing stability studies. As a result, three possible sources of Oxox were purified further: cabbage, carrot and mint leaves. Prior to the further purification of the enzyme from these sources, studies were conducted to determine the presence of Oxox in different varieties of commercially purchased and cultivated mint and cabbage leaves. The assays performed led to unexpected variational

differences, with the cultivated garden variety of cabbage leaves and commercially purchased Spanish mint leaves possessing elevated Oxox activities, demonstrating the presence of Oxox within a particular source was dependant upon location and cultivation medium.

The purification of cabbage, carrot and mint leaves was pursued by thermal treatment at 60 °C for 10 minutes, whereby Oxox was found to be thermally stable. The thermally treated extracts were concentrated using 20 %, 40 % and 60 % ASF. The concentration of the three extracts led to almost complete loss of the Oxox activity after ASF and dialysis. Initially these finding were unexplainable, however, further studies undertaken during this research identified the cause of this loss.

The purification of Oxox in cabbage, carrot and mint leaves was pursued further by cation exchange chromatography. Oxox within these sources did not bind to the carboxymethyl (CM) groups within the CM cellulose cation exchange media. Since Oxox in cabbage, carrot and mint leaves were exhibiting dissimilar ionic properties to Sigma and barley root Oxox, the molecular mass of Oxox in cabbage leaves was determined by gel filtration chromatography. The Sephacryl S-200-HR column was standardised using molecular mass standards ranging from 12.4 kDa to 200 kDa. The purification of cabbage leaves Oxox using Sephacryl S-200-HR led to the elution of Oxox between 510 mL and 560 mL, whereas the smallest molecular marker (Cytochrome c 12.4 kDa) eluted after 360 mL. These studies demonstrated cabbage leaves Oxox was significantly smaller than 12.4 kDa. Considering that the component isolated from cabbage leaves was substantially smaller than 12.4 kDa, concerns arose whether the oxalate oxidising components isolated from cabbage, carrot and mint leaves were in fact Oxox.

To further investigate the molecular mass of the oxidative components in cabbage, carrot and mint leaves, dialysis was performed using dialysis tubing with a molecular cut off of 12 kDa. Assay performed after dialysis identified that the oxidative components diffused out of the dialysis membrane, further suggesting the molecular mass of the oxidative components were less than 12 kDa. This data also aided the interpretation of earlier results obtained after ASF where Oxox activity in cabbage, carrot and mint leaves was undetectable after dialysis. To confirm the molecular mass of the oxidative components in the three sources, Sephadex G25, G15 and G10 chromatography were performed. This led

to the elution of Oxox along with the salt components, confirming Oxox in cabbage, carrot and mint leaves were less than 700 Da. As a result, the oxalate oxidative components were characterised and researched further, an overview of the procedures undertaken are given below:

7.3.1 Ionic Properties

The ionic properties of the oxidative components in cabbage, carrot and mint leaves were investigated since these components did not bind and interact with the CM groups within the CM cellulose cation exchanger. Diethylaminoethyl (DEAE) cellulose anion exchange media was employed to determine if the oxidative components were strongly negatively charged. The oxidative components did not interact with the DEAE groups, therefore it was concluded the oxidative components in the three sources were not strongly positively or negatively charged.

7.3.2 Staining Properties

Protein content was monitored throughout this research using Lowry and Bicinchoninic acid (BCA) protein assays. The cabbage, carrot and mint extracts analysed using these protein quantification assays led to positive results, whereby the protein content in each extract was measured successfully. However sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) led to the inability to visualise the active oxalate oxidative components on the polyacrylamide gel. The Lowry and BCA assays formed copper-protein complexes by interacting with functional groups within the analyte. It was also subsequently found that pyrroloquinoline quinine (PQQ) gave a positive result after analysing with BCA.

7.3.3 Spectral Characteristics

Chromatographic analysis undertaken throughout this research successfully detected the oxalate oxidative components at 280 nm, suggesting the components possessed aromatic properties. The cabbage and mint oxidative components were analysed using UV/Vis spectroscopy to determine their absorption capability. Cabbage and mint leaves oxidative components absorbed between 255 nm to 260 nm and 310 nm to 315 nm, coinciding with its detection at 280 nm during chromatographic studies.

7.3.4 Thermal Properties

Extensive thermal treatment at 60 °C for 20 minutes and 100 °C for 60 minutes was undertaken to identify the extent of the thermal stability of the oxidative components in the three sources. The oxalate oxidative activity remained intact after thermal treatment for 60 minutes at 100 °C, strongly suggesting the oxalate oxidative components in cabbage, carrot and mint leaves were not proteins or polypeptides. These finding suggested that the plant extracts possibly do not possess isolated aromatic groups, which would be expected in proteins, however were likely to be a conjugated organic molecules.

7.3.5 Electrospray Mass Spectroscopy

Positive and negative-mode electrospray mass spectroscopy (ESI MS) of cabbage and mint leaf oxalate oxidising components performed led to the visualisation of numerous fragments. Direct comparisons could not be made since the extracts were isolated using dissimilar protocols and differed in purity.

7.4 COFACTOR

The possibility of the oxalate oxidative components being a cofactor was investigated since the active components were not found to be proteins. It was suggested the components may have dissociated from their host protein during purification procedures. Most cofactors work in conjunction with host enzymes, however, PQQ, can function independently and is isolated using ion exchange chromatography. Since the oxalate oxidative components eluted with salts during Sephadex chromatography, it was proposed that the components of interest isolated from the three sources may be PQQ which had been isolated from its host protein in the presence of salts. PQQ has a molecular mass of 330 Da, is a conjugated molecule and possess an amine group, these characteristics were also determined in cabbage and mint oxalate oxidative components.

Given that PQQ is both highly conjugated and aromatic, it was expected to absorb at 280 nm during UV/Vis spectrometry, however, it absorbed at 217 nm, 252 nm, 323 nm and 357 nm. It was proposed PQQ had undergone a bathochromic shift due to the presence of methanol in the preparation which may have caused the 280 nm spectral band to shift to a longer wavelength changing in the extent of conjugation of PQQ.

Considering PQQ also contains an amine group, it formed a copper-amine complex during BCA preotein assays. These finding coincided with those obtained during protein quantification studies undertaken with the oxalate oxidative components.

The analysis of PQQ using negative-mode ESI MS was very successful since the resultant fragments were identified, however, positive-mode ESI MS analysis was more complex. Comparison of the cabbage and mint spectra after positive and negative-mode ESI MS could not be made with the spectra obtained with PQQ.

It can be concluded that cabbage and mint oxalate oxidative components are dissimilar, moreover, it was also determined that they were not PQQ.

7.5 OVERALL CONCLUSION

To conclude, there is vast amount of information available on the purification, properties and characteristics of Oxox, all of which differ. This research questions the reliability of the published data since some sources of Oxox have been isolated and purified using basic purification procedures such as subcellular fractionation and ASF only [297, 298, 292, 296, 305, 211, 7, 5, 60, 12, 9]. It is not feasible to identify the nature of a component by merely performing these two relatively basic procedures. If this was possible, then this research would have led to the identification of numerous 'sources' of Oxox since the screening process identified high oxidative activity in many sources.

There is no doubt the crops from the *Poaceae* family such as wheat and barley do in fact possess Oxox, however, the occurrence of Oxox in other sources such as banana peel is disputable. In addition to this, it is not clear from the literature whether – oxalate assays have been conducted identifying the 'true' activity of many published sources. Therefore, the great difference in the reported properties and characteristics of purified Oxox may be a reflection on whether the components isolated were in fact Oxox, hence, questioning the reliability of the data.

It was determined that cabbage and mint oxalate oxidative components were dissimilar, even though they both oxidise oxalate. This is not surprising since there are many reported

sources of Oxox with differing properties, including molecular mass and subunit composition, yet they still oxidise oxalate.

This research has led to the partial fulfilment of the aims set out in chapter 1.3. A novel sensor was developed using Sigma and barley root Oxox to detect oxalate for diagnostic use. The purification of an alternative source of Oxox has been more challenging than expected with novel oxalate oxidative components being isolated from cabbage, carrot and mint leaves which successfully detect oxalate.

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