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The immunoaffinity purification of human xanthine oxidase

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THE IMMUNOAFFINITY PURIFICATION OF

HUMAN XANTHINE OXIDASE

Submitted by

RICHARD BRYANT

For the degree of PhD of the

University of Bath

2003

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R. B. Bryant
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Finally, I could not have completed this PhD without the help of my family, especially my Parents. I am forever in their debt; to them I dedicate this thesis.

It's been fun.
The enzyme Xanthine Oxidase (XO) is the terminal enzyme of purine degradation in humans. However, it has become the focus of attention due to its ability to generate reactive oxygen species (ROS), and has been implicated in the pathogenesis of disease states. Although XO from sources such as bovine milk and rat liver have been studied extensively, information regarding XO from humans is less comprehensive. However, recent improvements in purification of the enzyme from human milk have led to an increase in attention devoted to this form. Human milk XO is considerably less active toward traditional purine substrates than enzyme purified from bovine milk. Furthermore, purified human liver XO has specific activity similar to the bovine enzyme, suggesting a possible tissue-dependent role for XO. The occurrence of the enzyme in human heart has not been conclusively determined although most reports allude to a possible low activity enzyme present in small amounts. This thesis describes the purification and partial characterisation of human heart XO, using anti-(HXO) polyclonal antibodies.

XO was purified from human milk and used as the immunogen to generate anti-(HXO) pAb in rabbits. Following purification, this antibody was used to determine an asymmetrical localisation of XO on the outer-surface of cultured human endothelial and epithelial cells. Such specific distribution suggests involvement of the ROS generating enzyme in cell-cell signalling.

The anti-(HXO) pAb was immobilised on a protein A-CNBr-activated Sepharose 4B matrix. Having identified 35 mM diethylamine as the most appropriate eluant, crude preparations of XO were obtained from post-mortem human liver and diseased heart homogenates by immunoaffinity purification. The specific activity of the heart enzyme toward pterin was determined to be 0.379 nmol/min/mg, considerably lower than XO purified in identical fashion from human liver (305.7 nmol/min/mg).
In conclusion, a reusable method for purification of tissue XO has been developed and used to obtain XO with very low specific activity from human heart. The role of this enzyme is discussed.
ABBREVIATIONS

A<sub>b</sub>  Absorbance at wavelength bnm
ADP  Adenine diphosphate
AMP  Adenine monophosphate
ATP  Adenine triphosphate
BSA  Bovine serum albumin
BXO  Bovine xanthine oxidase
CN (solution)  4-chloro-1-naphthol
CNBr-  Cyanogen-bromide activated
DMSO  Dimethylsulphoxide
DTT  Dithiothreitol
EDTA  Ethylenediaminotetra-acetic acid
ELISA  Enzyme-linked immunosorbent assay
Fab  Antigen-binding fragment of immunoglobulin molecule resulting from papain digestion
FAD  Flavin Adenine dinucleotide (oxidised)
FADH<sub>2</sub>  Flavin Adenine dinucleotide (reduced)
Fc  Crystallisable, non-antigen binding fragment of immunoglobulin molecule resulting from papain digestion
Fe/S  Iron-sulphur redox centre
g  Gravitational force
HRP  Horse-radish peroxidase
HXO  Human xanthine oxidase
Ig  Immunoglobulin
IgG  Immunoglobulin class gamma
IgM  Immunoglobulin class mu
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Kda</td>
<td>Kilodalton(s)</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram(s)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram(s)</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibodies</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline with 0.1% (v/v) Tween-20</td>
</tr>
<tr>
<td>PFR</td>
<td>Protein:flavin</td>
</tr>
<tr>
<td>pH</td>
<td>(-\log_{10} (\text{hydrogen ion concentration}))</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline containing 0.05% (w/v) Tween 20</td>
</tr>
<tr>
<td>Temed</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) amino methane</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram(s)</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per unit volume</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
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Chapter 1

General introduction
1. General Introduction

In 1981 Granger, Rutili and McCord proposed the concept that Reactive Oxygen Species (ROS) play a leading role in producing the microvascular dysfunction associated with the reperfusion of ischaemic tissue. The source of the ROS was assumed to be the enzyme xanthine oxidase (XO), largely because allopurinol, an inhibitor of its activity, provided protection against ischaemia-reperfusion injury. This hypothesis has led to extensive studies into XO, the ultimate goal being to elucidate its precise physiological function given that it appears to play a role in purine catabolism, detoxification of xenobiotics and has an antioxidant capacity by producing urate. Bovine milk, and rat and chicken liver XO have been well characterised. However, relatively little information is available concerning the enzyme from human tissues.

1.1. Enzymology

Xanthine oxidase is a homodimer. Each 150 kDa subunit is catalytically independent and contains a full complement of redox active sites, comprising one molybdenum, a non-covalently bound FAD group; and two iron-sulphur centres. Associated with the molybdenum centre is a pterin cofactor, which is co-ordinated to the Mo molecule via two side chain thiols. The two iron-sulphur centres are of the 2Fe/2S spinach ferrodoxin type. The reductive and oxidative half reactions of the catalytic cycle are spatially separated and electrons are shuttled around the redox centres facilitating the process (Massey et al., 1969; Hart et al., 1970; Rajagopalan and Johnson, 1992; Hille, 1996) (Figure 1.1).
Figure 1.1 The redox centres of xanthine oxidase.

Previous studies have shown that, apart from NADH, which acts at the FAD site, all reducing substrates interact at the molybdenum site of the enzyme (Bray, 1975). Electrons are then passed via the FAD centre to oxygen or NAD$^+$ (in vivo), or via the Fe:S centres to a range of artificial acceptors such as methylene blue (in vitro). Reduction of molecular oxygen leads to generation of hydrogen peroxide and superoxide anion, ROS, which have been implicated as causative agents in a range of disease states, especially ischaemia-reperfusion.
1.1.1. **PURINE CATABOLISM**

The best known role of XO is its involvement in purine catabolism, where it serves as the rate limiting enzyme through which all purines are channelled for terminal oxidation (McCord, 1985) (Figure 1.2). Like all molybdenum enzymes, water serves as the source of oxygen to be incorporated into the substrate, rather than molecular oxygen. The enzyme catalyses the hydroxylation of xanthine at the C(8) position (Hille, 1996). In primates the product, urate, is excreted, whereas in most other mammals it is further degraded to allantoin by urate oxidase.

![Diagram of Purine Catabolism](image)

*Figure 1.2 The involvement of xanthine oxidase in purine catabolism.*

Xanthine oxidase actually has a dual role, regulating degradation of (hypo)xanthine, and also controlling the balance between degradation and salvage of purines (Kaminski and
Jezewska, 1979). It has been estimated that only 10% of all purines are degraded to urate, the majority being reused via the salvage pathway (Stryer, 1996).

Urate is thought to scavenge singlet oxygen and hydroxyl radicals (Ames et al., 1981) as well as peroxynitrite (Squadrito et al., 2000). Higher primates lack urate oxidase which, coupled to an active reabsorption mechanism in the kidney, results in high plasma urate concentrations compared to other animals. Therefore, strong evolutionary advantages may be conferred upon higher primates, as urate could scavenge the ROS responsible for aging, cancer or oxidative stress in the central nervous system (Hooper et al., 2000; Becker, 1993; Ames et al., 1981; Harrison, 2002).

Xanthinuria is the term given to the disorder where individual humans genetically lack XO. This condition is rare and the individuals appear little the worse for the deficiency, the only clinical manifestation observed being the appearance of xanthine stones in some patients. Furthermore, in conditions such as gout, inhibition of XO with the specific inhibitor allopurinol (4-hydroxypyrazolo (3,4-d) -pyrimidine), is beneficial, appearing to have few side effects (Bray, 1975).
1.1.2. **INTERCONVERSION BETWEEN D AND O FORMS OF XO AND ENZYME STRUCTURE**

Xanthine oxidase from non-mammalian sources, ranging from bacteria to avian liver, reacts only poorly with oxygen, especially when NAD$^+$ is also present as an alternative acceptor, and appears to exist in a single stable form. However, enzymes from mammalian sources exist in two partially interconvertible forms. In the dehydrogenase (D) form, NAD$^+$ is the preferred electron acceptor, whilst the oxidase (O) form prefers oxygen as the acceptor (Hille and Nishino, 1995) (Figure 1.3).

![Diagram of interconversion between D and O forms of xanthine oxidase](image)

*Figure 1.3 The (partial) interconversion between the D and O forms of xanthine oxidase.*
In vitro conversion of the D to the O form can be brought about by a variety of conditions such as anaerobiosis, storage at -20°C or heating at 37°C, as well as by the use of sulfhydryl-oxidising reagents (Stirpe and Della Corte, 1969). The in vivo enzyme is found predominantly (approximately 80%) in the D form (Waud and Rajagopalan, 1976), and reversible conversion can be effected by the use of sulfhydryl reagents (McKelvey et al., 1988). A disulphide bond formation involving residues Cys 535 and Cys 992 is thought to be central to this (Rasmussen et al., 2000). Alternatively, irreversible conversion of D to O form can result from cleavage of a 20kDa fragment from the D form of the enzyme (McCord, 1985), although merely a "nick" of the polypeptide has also been postulated to result in the shift to the irreversible O form (Nishino and Tamura, 1991). While the 20 kDa fragment cleaved is not essential for oxidase activity, it contains 10 free sulfhydryls which are implicated in maintaining the conformation required to bind NAD* in the D form (Waud and Rajagopalan, 1976).

Stirpe and Della Corte (1969) reported that the xanthine-oxidising activity in rat liver homogenates was largely attributable to the NAD* dependent dehydrogenase form. On purification without the use of a thiol reagent, the enzyme had dehydrogenase to oxidase ratio of 2.7-3.0, (Waud and Rajagopalan, 1976). Ikegami and Nishino (1986) avoided the use of thiol reagents during purification of rat liver enzyme, as they were found to inactivate XO during prolonged incubation. Although the dehydrogenase to oxidase activity ratio of their purified enzyme was low, incubation with dithiothreitol converted it to the NAD*-dependent form (Ikegami and Nishino, 1986).

Following treatment of rat liver enzyme with trypsin, incubation with guanine-HCl and subsequent HPLC, three peptide fragments were obtained (Amaya et al., 1990). It was proposed that the FAD is associated with the 40 kDa fragment, the two iron-sulphur centres with the 20 kDa, and the molybdopterin with the 85 kDa fragment. Although these centres are well separated in the sequence, the apparently rapid electron transfer between them suggests that they are in close proximity in the native protein (Amaya et al., 1990). The
amino acid sequences of rat liver and *Drosophila* enzyme were compared in this study, and the sequences were not well conserved around the NAD\(^+\) binding sites. During the tryptic digest the rat liver enzyme had been irreversibly converted to the type O form. It was proposed that the three tryptic peptide fragments, although not dissociated from each other at this point, had re-orientated themselves, resulting in conformational changes in the environment around the flavin, decreasing the binding affinity for NAD\(^+\). It was suggested that the environment of the NAD\(^+\) might be one of the reasons why the D to O conversion of XO occurs only in mammalian enzymes (Amaya *et al*., 1990).

Crystal structures of the O and D forms of bovine milk XO were recently elucidated (Enroth *et al*., 2000). These researchers discovered that cleavage of surface loops of the D form on conversion to O form results in the major structural rearrangement of another loop close to the flavin ring (Gln 423-Lys 433). Consequently, access of the NAD\(^+\) substrate to the FAD cofactor is prevented and accompanying changes the electrostatic environment of the active site result in the switch of substrate specificity.

The genes for mouse and human XO have been isolated and characterised (Cazzaniga *et al*., 1994; Xu *et al*., 1996). The exon-intron structure is highly conserved between these, suggesting a common ancestral gene. Chicken liver XO sequence corresponds to 1358 amino acids (Sato *et al*., 1995), whereas the mammalian enzymes range from 1330-1335. The amino acid sequences of the 'high' activity human liver and intestine enzymes (Ichida *et al*., 1993; Saksela and Raivio, 1996; Yamamoto *et al*., 2001) and 'low' activity human mammary gland XO have been shown to be essentially identical (Pearson, 2001). Furthermore, the bovine milk XO cDNA sequence shows 90% homology with human liver enzyme (Abadeh *et al*., 1992a; Ichida *et al*., 1993; Hille and Nishino, 1995; Berglund *et al*., 1996) and also with rat (Amaya *et al*., 1990), mouse (Terao *et al*., 1992) and cat XO (Tsuchida *et al*., 2001). A weaker homology exists between the mammalian enzyme and *Drosophila* XO (Keith *et al*., 1987; Riley, 1989)
The elucidation of the crystal structure of aldehyde oxidase (MOP) from Desulfovibrio gigas (Romao et al., 1995) resulted in major advances in the understanding of XO. An example of this is the determination of the nature of the two iron-sulphur centres. The N-terminal domain of XOR contains eight cysteine residues, which have been shown to be ligands to the two Fe$_2$S$_2$ groups. However, contrary to original thinking, only the N-terminal cysteines bind one of the Fe$_2$S$_2$ groups in the regular plant-type ferrodoxin motif. The second Fe$_2$S$_2$ group is bound by the remaining four cysteines in an unusual protein fold, unique to the XO group of enzymes, which include MOP, carbon monoxide dehydrogenase (CODH) from Oligotropha carboxidovorans and aldehyde oxidase (MOD) from Desulfovibrio desulfuricans (Harrison, 2002). Mutagenesis studies have now led to the assignment of Fe/S II and Fe/S I to the N-terminal and C-terminal clusters respectively (Iwasaki et al., 2000).

Analysis of the crystal structure of bovine milk XO has confirmed the earlier results gained from proteolytic cleavage of XO. The N-terminal 2 Fe$_2$S$_2$ cluster (1-165) and FAD domain (226-531) are separated by an interconnecting peptide (166-225). The FAD domain is also connected to the C-terminal Mo-co site (590-1332) via another disordered segment (532-589). The respective distances between the Mo, Fe/S I, Fe/S II and FAD centres in the tertiary structure have been determined to be 14.7 Å, 12.4 Å and 7.8 Å (Enroth et al., 2000).
1.1.3. INACTIVE FORMS AND THE REGULATION OF XO

Three inactive forms of XO exist. Two of these, demolybdo and desulpho occur naturally and manifest themselves as dysfunctionality at the molybdenum active site (Bray, 1975). The demolybdo form lacks the molybdenum atom, and possibly also the associated pteridine cofactor (Ventom et al., 1988). The second inactive form, desulpho XO, has the Mo=S group, essential for activity, replaced by Mo=O (Bray, 1975). Activity toward xanthine and other reducing substrates acting at the molybdenum site in both the demolybdo and desulpho forms is compromised.

Bovine and human milk both contain these inactive forms, albeit in differing amounts. Bovine milk XO contains approximately 65% of the "theoretical" Mo, the contribution from sulpho enzyme enabling the enzyme to achieve 40% functionality toward xanthine (Bray, 1975). A study by Abadeh et al. (1992) on XO purified from human milk indicated, that although NADH oxidation occurred at a similar rate to that shown by the bovine enzyme, its activity involving xanthine as reducing substrate was only 1-6% that of its bovine counterpart (Abadeh et al., 1992b). Godber et al. (1997) revealed that human milk XO is at least 95% demolybdo. Of the 5% molybdo, approximately 80% is desulpho, rendering the enzyme only about 1% active toward xanthine (Godber et al., 1997).

It has also been established that rat liver XO is approximately 50% inactive in both crude and purified samples, demolybdo form accounting for 15% and 10% of the crude and purified enzyme respectively (Ikegami and Nishino, 1986). This difference was thought to be due to heat treatment in the purification procedure, possibly denaturing demolybdo enzyme. The predominant inactive form of the rat liver XO was found to be desulpho.

The existence of inactive forms suggests a physiological significance. Knowledge of sulphur-incorporating enzymes, such as rhodanese (Coughlan, 1981), gives insight into the possible post-translational modification of XO. Indeed desulpho-sulpho conversion of
bovine milk XO, with concomitant activation toward xanthine, has been demonstrated in vitro (Wahl and Rajagopalan, 1982). Activation of XO in milk from mothers during the first few weeks post partum (Brown et al., 1995) is too great to be explained solely by a desulpho/sulpho mechanism (Harrison, 1997), and may give credibility to a possible, albeit cumbersome, Mo-incorporated activation. There is evidence of XO activation in mouse L929 cells by the addition of Mo (Falciani et al., 1994), however repetition of this has proved unsuccessful in cultured human cells (Page et al., 1998).

Furth-Walker and Amy (1987) measured the effect of diet on XO activity. Following the administration of a low protein, iron or vitamin E deficient diet to rats, it was found that although the number of detectable protein molecules and mRNA copies of XO did not alter, its activity did. This led to the conclusion that diet induces post-translational changes. Furth-Walker and Amy (1987) suggested that a sulphur-incorporated activation is under genetic control, and that desulpho XO is a physiologically significant intermediate in the formation of active enzyme (Furth-Walker and Amy, 1987).

Xanthine oxidase activity is also subject to regulation at the transcriptional level. The expression of the XO gene in humans is significantly lower than in mice, and both transcription rates and core promoter activity of the gene are repressed (Xu et al., 2000). Xu and co-workers identified repressor and activator binding regions responsible for regulating core promoter activity in hepatocytes and vascular endothelial cells. They proposed a model involving interaction of E-box and TATA-like elements to account for this restricted gene activity.

Possible (patho-) physiological upregulation of XO by interferons (IFNs) in rodents has been demonstrated. XO activity in mice was increased as a result of administration of either interferons (IFN α and β, leukocyte IFN) or IFN-inducers (lipopolysaccharide, poly I,poly C, tilorone) (Ghezzi et al., 1984). IFNγ induced XO activity in vivo in rat lungs and was shown to increase transcription of the XO gene, rather than increased translation of XO mRNA in
vitro (Dupont et al., 1992). In cultured human cells an increase in enzymic activity in response to IFNγ was also seen (Page et al., 1998). In this case an increase in specific mRNA and XO protein of 2-3 fold was observed, which corresponded to an 8 fold increase in specific activity, providing further evidence of a post-translational activation. None of these studies observed an alteration of D/O ratio accompanying the cytokine induced activation (Ghezzi et al., 1984; Dupont et al., 1992; Page et al., 1998).

Xanthine oxidase activity in vivo may also be hormonally controlled. Adult male rat livers have been shown to have twice the XO activity of female rat livers (Levinson and Chalker, 1980), while in men, there is 20% higher liver XO activity compared to females (Guerciolini et al., 1991). As result, it was suggested that androgens upregulated XO activity, whilst oestrogens have an inhibitory effect (Levinson and Chalker, 1980; Levinson and Decker, 1984; Beedham, 1985). It was proposed that this increase was regulated at the transcriptional level (Schieber and Edmondson, 1993). In cultured mouse mammary epithelial cells, lactogenic hormones have been found to regulate milk protein synthesis (McManaman et al., 2000). Prolactin and cortisol were found to elevate XO mRNA levels, increase de novo synthesis and decrease degradation of XO protein. It was suggested that a MAP kinase-dependent pathway mediates the effects of the lactogenic hormones on XO.

Hypoxia-induced increased XO activity has been demonstrated in cultured bovine and rat endothelial cells (Hassoun et al., 1994; Poss et al., 1996; Kayyali et al., 2001) and in rat brain slices (Batelli et al., 1998). The observed increase in rat epididymal fat pad activity was due to an increase in XO mRNA (Hassoun et al., 1994). Conversely, XO activity increases in cultured bovine aortic endothelial cells (Poss et al., 1996) and rat brain slices (Batelli et al., 1998) were attributed to post-translational modifications. The hypoxia-induced post-translational activation of XO in rat pulmonary microvascular endothelial cells observed by Kayyali and colleagues (2001) was found to be via protein phosphorylation. Phosphorylation of XO after 4 hours of hypoxia, was accompanied by a 2-fold increase in XO activity, the mechanism involved p38 kinase and casein kinase II. However, a complex
pre-and post-translational mechanism has been cited for the increase in activity observed during hypoxia in cultured 3T3 fibroblasts (Terada et al., 1997). The means of control for this is thought to be via hypoxia inducible-factor-1 (HIF-1)-like sites present in the 5' upstream region of mouse and human genes (Terada et al., 1997; Hoidal et al., 1997).

A third inactive form of XO, deflavo-enzyme, lacks the FAD cofactor. This form does not appear to occur naturally, but because the flavin moiety is non-covalently bound, it is susceptible to removal under a number of conditions. In 1969 Komai and co-workers reported the first successful removal of FAD in a reversible manner. It involved treatment of XO with high CaCl2 concentrations (e.g. 2M) (Komai et al., 1969). Later this work was repeated by the group of Kanda using 3M KI (Kanda et al., 1972). The recovery of initial activity by these groups on incubation of deflavo enzyme with FAD, was about 60% and 80% respectively. Deflavo enzyme will also result from the selective proteolysis produced via incubation with subtilisin at high pH (Nagler and Vartanyan, 1976) and following alkylation of the FAD by treatment with iodoacetamide or phenylacetate ((McGartoll et al., 1970; Bray, 1975).

The removal of, and the subsequent retrieval of activity following incubation with FAD, is a property that has been exploited when analysing the kinetic characteristics of XO. In 1989 Saito et al. found that deflavo XO, generated via incubation with calcium chloride, could be reconstituted in the presence of FAD to produce enzyme with 85% of the original activity intact, all of which was the O2-dependant type. The D to O conversion could largely be prevented when dithiothreitol was included during incubation. The group also suggested that DTT-induced reduction of disulphides to the sulfhydryl groups responsible for the D to O conversion was possibly more effective on the deflavo-apoenzyme, than on reconstituted enzyme (Saito et al., 1989).

The FAD site has been probed in chicken liver xanthine dehydrogenase using the NAD+ analogue 5'-[p-(fluorosulfonyl)]benzoyl]adenosine (5'-FSBA) (Nishino, 1987; 1989). This
analogue reacts with tyrosine residues and the result of its action with D form enzyme was the loss of xanthine-NAD$^+$ activity. However, the xanthine-O$_2$ reactivity was unaffected by this covalent modification, allied with accompanying spectral changes, suggests the reactive tyrosine residue plays a specific role in binding NAD$^+$ (Hille, 1996).

The complexity of the enzyme, as discussed here, has hindered the expression of active recombinant enzyme. Nishino and co-workers reported expression of rat liver XO in the baculovirus-insect cell system producing a recombinant enzyme of identical size to the native form. However, the enzyme comprised a mixture of demolybdo- and other inactive forms (Nishino et al., 1997; 1999). Transient expression of human XO in COS-1 cells has been effected by Saksela and colleagues (1996). Although XO activity was demonstrated in cell lysates, the authors gave no indication of the content of inactive forms. More encouragingly, recent studies have reported the expression of Drosophila XO in Aspergillus nidulans, with specific activity of the recombinant XO being indistinguishable from that of the enzyme purified from fruit flies (Adams, 1997; 2002).
1.2. THE PATHOGENESIS OF ISCHAEMIA-REPERFUSION DISORDERS

The involvement of D to O conversion of XO and subsequent generation of Reactive Oxygen Species (ROS) in ischaemia-reperfusion injury was proposed by Granger and co-workers (Granger et al., 1981) (Figure 1.4).

During ischaemia, the limited blood flow to a tissue results in a state of hypoxia. Oxygen is required for ATP production and consequently the cell's energy charge falls. A result of this is that calcium ions leak into the cytosol, activating a protease [perhaps with the involvement of calmodulin (Granger et al., 1986)] that converts the D to the O form.

Figure 1.4 The mechanism of ROS production by Xanthine Oxidase during Ischaemia-Reperfusion (from (Granger et al., 1981)).
Additional to the formation of the O form, there is a concomitant depletion of the cell’s ATP, which is catabolised eventually to hypoxanthine, a purine substrate for XO.

During reperfusion, molecular oxygen returns to the tissue and a burst of ROS is produced. The ROS, namely superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), are generated by one electron and two electron reductions respectively. Superoxide dismutase (SOD)-catalysed disproportionation of O$_2^-$ will also yield H$_2$O$_2$. Both species are only moderately active, although H$_2$O$_2$ is able to freely cross biological membranes (Halliwell and Gutteridge, 1986). A more reactive oxidant, the hydroxyl radical (HO$^-$), is formed from the O$_2^-$ driven “Fenton-type” reaction which also requires H$_2$O$_2$. The overall result is summarised in equation (Figure 1.5).

\[
\text{Fe}^{III} + \text{O}_2^- \longrightarrow \text{Fe}^{II} + \text{O}_2 \quad \text{(Equation 1.1)}
\]
\[
\text{Fe}^{II} + \text{H}_2\text{O}_2 \longrightarrow \text{Fe}^{III} + \text{HO}^- + \text{HO}^- \quad \text{(Equation 1.2)}
\]

Net Balance (Haber-Weiss Reaction):
\[
\text{O}_2^- + \text{H}_2\text{O}_2 \longrightarrow \text{O}_2 + \text{HO}^- + \text{HO}^- \quad \text{(Equation 1.3)}
\]

**Figure 1.5** The Fenton Reaction, leading to the formation of the hydroxyl radical.

The interaction of H$_2$O$_2$ with reduced iron to produce HO$^-$ is referred to as the Fenton reaction. Interaction of O$_2^-$, ferric iron and H$_2$O$_2$ to produce HO$^-$ in biological systems is known as the iron-catalysed Haber-Weiss reaction. The Haber-Weiss reaction can involve chelates of other transition metals such as Cu. Relevant chelates of Fe include low
molecular weight chelates with adenosine diphosphate such as ADP-Fe$^{3+}$, citrate-Fe$^{3+}$, and certain amino acid chelates of Fe$^{3+}$. Superoxide is also known to reduce iron-loaded ferritin, transferrin and lactoferrin, releasing Fe which may participate in the Fenton reaction (Chen et al., 1997). The damage caused by the HO\textsuperscript{•} may be site specific if the metal ion or its complex are bound to the target, or non-site specific if HO\textsuperscript{•} is generated in the bulk solution (Cadenas, 1997).

HO\textsuperscript{•} is highly electrophilic and, if formed near DNA, can add to DNA bases or abstract hydrogen atoms from the helix (Pryor, 1988). In proteins, histidine is an important metal binding site and can therefore help localise damage initiated by peroxides (Stadtman, 1990a; 1990b; Davies et al., 1987). Both structural and enzymic proteins are susceptible to radical attack, thiol groups being particularly vulnerable. The resultant disulphide formation leads to protein deformation or inactivation of enzymes, for example glutamine synthetase (Tappel, 1965; Chio, 1969; Rivett, 1990).

Damage can also occur to extracellular proteins and glycosaminoglycans such as collagen and hyaluronic acid, respectively, and lead to the loss of structural integrity of these molecules (Fridovich, 1978). Free radical damage may also be in the form of peroxidation of cell membrane polyunsaturated lipids, forming unstable hydroperoxides, or endoperoxides, ultimately leading to extensive membrane destruction. Overall, ROS are thought to be responsible for mediating ischaemia-reperfusion (I/R) injury to a range of tissues including intestine, liver, heart and joints (Sussman and Bulkley, 1990).

The evidence for the attenuation of I/R induced injury by SOD, copper di-isopropyl salicylate (CuDIPS, a lipophilic SOD mimetic), catalase (CAT) and dimethylsulphoxide (DMSO) is considerable (Parks and Granger, 1983; Granger et al., 1986; Hearse et al., 1986; Granger, 1988). Originally, it was believed that the beneficial effects resulted from prevention of secondary oxidants, i.e. HO\textsuperscript{•}, generated during reperfusion via a Fenton type reaction, as described earlier (Figure 1.5). However, DMSO, although a hydroxyl radical
scavenger, has other biological effects, including the ability to inhibit neutrophil adherence (Sekizuka et al., 1989). Furthermore, Suzuki and co-workers proposed that SOD and CAT inhibited the adherence of PMNs to post-capillary venules in vivo (Suzuki et al., 1989; 1991). It may be tempting to explain I/R injury solely by the generation of superoxide by neutrophils. However, this does not explain how XO inhibition can prevent injury (Sussman and Bulkley, 1990).

The decrease in ATP and increase in hypoxanthine during ischaemia is well documented ((Schoenberg et al., 1985; Blum et al., 1986). The first of these studies demonstrated that an ischaemic period of 2 hours reduced the ATP concentration by 40% of the pre-ischaemic value. It was also found that the depletion in ATP concentration was associated with increase in tissue concentrations of AMP (8 fold), hypoxanthine (10 fold) and uric acid (4 fold). Hypoxanthine concentration in normal perfused intestinal mucosa is approximately 20 μM, rising to greater than 200 μM during ischaemia (Schoenberg et al., 1985). Studies on rat intestine by (Blum et al., 1986) demonstrated the rapid and complete depletion of mucosal ATP within 20 minutes of total ischaemia. Longer ischaemic periods failed to reduce ATP levels further.

Relatively little is known about the mechanisms and kinetics of the D to O conversion. An increase in cytosolic Ca²⁺ during oxidative stress has been demonstrated (Dreher et al., 1995). It was thought to originate from intracellular stores, possibly as a result of inactivation of the ER Ca²⁺-ATPase, leading to the subsequent influx of extracellular Ca²⁺. However, there is direct evidence against the involvement of the main calcium-activated proteases, calpains (Stark et al., 1989). An alternative hypothesis is that, under the reducing conditions and with the glutathione depletion found in ischaemic tissue (McKelvey et al., 1988), the bonds holding partially-proteolysed XO are broken, the 20kDa fragment is released and the D to O conversion follows (Sarmesto et al., 1996). Exact D/O ratios in tissue are hard to determine because of the likelihood of artifactual conversion induced during purification. However, it is now generally accepted that 10-20% of the enzyme exists.
in the O form in normal tissue (Parks et al., 1988; Lindsay et al., 1991; Marubayashi et al., 1991; Partridge et al., 1992; Brass, 1995; Kooij et al., 1994; 1995). Stringent precautions were taken by (Parks et al., 1988) when determining that only 50% of D is converted to O in rat bowel after 2 hours of ischaemia, representing a 2-3 fold increase in activity of the ROS producing form of the enzyme.

The D to O conversion required for the I/R model proposed by Granger is being viewed with increasing scepticism as it is doubtful it can happen within the time-scale required to produce the tissue damage observed during I/R (Engerson et al., 1987; Parks et al., 1988; Yokoyama et al., 1990; Frederiks et al., 1993; Tan et al., 1993; Wiezorek et al., 1994; Kooij et al., 1994; 1995; Frederiks and Bosch, 1996). Furthermore, as already stated (Section 1.1.3), increase in XO activity has been observed during hypoxia in studies of cultured bovine and rat endothelial cells (Hassoun et al., 1994; Poss et al., 1996; Kayyali et al., 2001) and in rat brain slices (Batelli et al., 1998). However, no D to O conversion was observed in any of these studies.

There are several lines of evidence suggesting that the damage attributed to I/R is largely inflicted during the resumption of blood flow, rather than being a delayed manifestation of injury sustained during ischaemia. The administration of SOD near the end of a 1 hour ischaemic period in the feline gut almost completely prevented the injury incurred during reperfusion (Granger et al., 1981). Parks and Granger in 1986 found that mucosal damage did occur in the feline gut during a 4-hour ischaemic period, without reperfusion. However, they also established that a 3-hour ischaemic period, followed by reperfusion with deoxygenated blood produced significantly less injury than reperfusion with oxygenated blood. Their overall conclusion was that, although damaged is incurred during the ischaemia, it is with reintroduction of blood that most injury occurs (Parks and Granger, 1986a).
1.2.1. **Evidence for the Involvement of XO in I/R: Inhibition Studies**

The involvement of XO in I/R has been investigated using inhibition studies. Administration of allopurinol, a specific inhibitor of XO, during I/R led to reduction in severity of intestinal mucousal lesions ((Parks et al., 1982; Schoenberg et al., 1985) and attenuation of increased microvascular permeability to plasma proteins (Granger, 1988). Allopurinol is a competitive inhibitor of hypoxanthine and xanthine, to which it is chemically similar to, acting at the molybdenum centre. Other inhibitors acting at this site include amflutizole (Werns et al., 1991) and BOF 4272 (Okamoto and Nishino, 1995).

Conflicting reports of the efficacy of allopurinol in limiting infarct size have been made. This is particularly evident in species in which the presence of heart XO has not been fully established. (Downey et al., 1987) reported that, in contrast to rats, infarct size and ROS production in rabbits were not limited by allopurinol administration. They concluded that XO was an important source of free radicals in rats, but not in rabbits. On the other hand, (Terada et al., 1991) demonstrated protection by allopurinol in the rabbit heart. Human patients undergoing coronary artery bypass surgery were also found to benefit from allopurinol administration (Johnson et al., 1991).

Allopurinol has also been shown to completely inhibit superoxide production in cultured human umbilical vein endothelial cells (HUVECs) (Michiels et al., 1992). The conclusion drawn was that XO is a primary producer of ROS after reperfusion, a finding complementing studies on endothelia in rat (Ratych et al., 1987) and cow (Zweier, 1988). However, allopurinol, as well as oxypurinol, has been shown to be a powerful hydroxyl radical scavenger in vitro, albeit at high concentrations (>700 μM), suggesting the benefits of its administration may not involve XO inhibition (Moorhouse et al., 1987).

Inhibition studies have used other inhibitors such as amflutizol () or BOF 4272( ). These inhibitors also block the molybdenum centre of XO. There is currently no specific inhibitor
of the NADH oxidase activity. It is strongly blocked by diphenyleneiodonium, a relatively non-specific inhibitor of flavoenzymes, including NADPH oxidase.

Preferential tungsten incorporation over molybdenum will also render XO inactive. When placed on a molybdenum-deficient, tungsten-rich diet, animals demonstrated a 75% reduction in intestinal XO activity and a corresponding amelioration of I/R-induced increased microvascular permeability. However, this approach is equivocal as other Mo-containing enzymes, such as aldehyde oxidase, are also inactivated by tungsten incorporation (Parks and Granger, 1986b). Activity of W-induced demolybdo- rat liver enzyme could not be restored by addition of molybdate in vitro. However, in vivo restoration could be achieved, even in the presence of inhibitors of protein synthesis (Johnson et al., 1974).
1.2.2. **THE NADH OXIDASE ACTIVITY**

The reportedly low activity of XO in human milk and tissue has led to speculation as to other physiological roles for the enzyme, and to questioning of its involvement in l/R injury. Another query concerns the time scale and extent of D to O conversion central to the Granger l/R model (Parks et al., 1988; Lindsay et al., 1991; Marubayashi et al., 1991; Partridge et al., 1992). NADH differs from other reducing substrates in donating its electrons directly to the FAD site (Bray, 1975). Consequently, NADH oxidation is not affected by variations at the molybdenum site. NADH oxidase activity has been demonstrated in XO from several sources, including bovine milk (Nakamura, 1991), chicken liver (Fonoll et al., 1980; Nishino, 1989) and turkey liver (Fhaolain and Coughlan, 1976).

Sanders et al. (1997) reported that the NADH oxidase activity of the D form of human milk enzyme, in the presence of molecular oxygen, was sufficient to produce superoxide anion at appreciable rates. The kinetics of the D forms of human and bovine XO were found to be very similar, even though the xanthine activities of these two enzymes differed markedly (Sanders et al., 1997). Conventional xanthine oxidase activity of human O form implicated in Granger’s mechanism for l/R injury was found to have a maximal superoxide production of 0.05 µmol/min/mg. In contrast, superoxide production due to its NADH oxidase activity is four times greater (0.2 µmol/min/mg), suggesting modification of the l/R hypothesis (Harrison, 1997; Figure 1.6).
Evidence supports the necessary ischaemic NADH accumulation (Williamson, 1966) whilst the lower pH observed during ischaemia (Falchuk et al., 1970) favours oxidation of the NADH activity, rather than xanthine (Harrison, 1997). The theory may also explain the ineffectiveness of allopurinol and oxypurinol at attenuating myocardial infarct size (Downey et al., 1987), as the NADH oxidase activity is unaffected by these inhibitors. There is currently no specific inhibitor of this activity. Diphenyleneiodonium will strongly block NADH oxidation, however it is a non-specific inhibitor of flavoenzymes in general, including NADPH oxidase (Stuehr et al., 1991; O’Donnell et al., 1994). This hypothesis, however, has not yet accounted for the potentially major inhibitory effects of high cytosolic NAD$^+$ concentrations seen under normoxic and ischaemic conditions.

Figure 1.6 The mechanism of ROS production by XO NADH oxidase activity during ischaemia-reperfusion (Harrison, 1997).
1.3. INVOLVEMENT OF ROS IN SIGNAL TRANSDUCTION

Currently there is widespread interest in the signalling mechanisms involving ROS. Putative roles for the oxidants include participation in processes as diverse as the control of vascular tone, carotid body function, thrombosis and proliferation (Wolin et al., 1997). Physiological stimuli alter the levels of specific ROS, which consequently act within cells in a second-messenger-like manner, and also in cell-to-cell tissue hormone-type signalling. In many cases the origin of the ROS involved in these processes is unknown although a candidate for their production is XO. Discussed here are two examples of the type of signalling, possibly involving XO-derived ROS, currently under investigation.

1.3.1. INFLAMMATION

A salutary effect of the activation of XO may be seen during acute inflammation. A possible cascade of events that may occur during inflammation, involving the production of ROS by XO, has been postulated (Dupont et al., 1992; Pfeffer et al., 1994). These ROS may be the signal transducers for the attraction and activation of neutrophils and upregulation of cytokines and their receptors. As mentioned previously (Section 1.1.3), upregulation of the activity of XO in response to inflammatory cytokines has been demonstrated (Ghezzi et al., 1984; Dupont et al., 1992; Page et al., 1998), although no alteration in D/O ratio was observed. However, (Friedl et al., 1989) found that pro-inflammatory cytokines and other mediators, including TNFα, IL3 and C5A all effected D to O conversion in endothelial cells in vitro. (Phan et al., 1992) demonstrated an increase in oxidase activity following incubation of endothelial cells with the neutrophil-derived protease, elastase. The perinuclear increase in levels of elastase in neutrophils during exocytosis may cause the D to O conversion, should the protease enter endothelial cells. This type of conversion is a strategy used by the body when a rapid response is required, there not being sufficient time to complete gene expression. The resultant increased ROS
production, along with TNFα and other cytokines may then activate the transcription factor, NF-κB, which binds to the nucleus and, in turn, upregulates the transcription of adhesion molecules, chemotactic molecules, and increases cell growth.

In 1997 Bulkley reported that the administration of TNFα to the media of a hybridoma-generated line of human umbilical vein endothelial cells, led to the expression of intercellular adhesion molecule-1 (ICAM-1) on the endothelial surface. He also described the attenuation of this expression via administration of cell-permeable antioxidants, or by the overexpression (4 fold) of human Cu-, Zn-SOD within the cells, suggesting that superoxide generation intracellularly appears to be the signal transducer in this mechanism. However, he emphasised that caution must be taken when extrapolating these findings to the in vivo situation. For example, injection of pathophysiological levels of TNFα in vivo (rat peritoneal cavity) led to the localisation of neutrophil adhesion primarily to the venular epithelium, suggesting that there are physiological differences in expression of adhesion molecules in situ, compared to in vitro (Morita et al., 1995; Bulkley, 1997).

Cytokines and their receptors will also be upregulated by NF-κB, thereby amplifying the inflammatory response. Therefore, I/R injury may be as a result of the aberrant activation of this normal reticuloendothelial cell function following the ischaemia induced D to O conversion (Bulkley, 1997). Further evidence supporting this is derived from studies on the feline small intestine. Ischaemia-reperfusion injury to it could be prevented by either neutrophil ablation (Hernandez et al., 1987) or by blocking neutrophil adherence to the microvasculature (Horie et al., 1996).

Xu et al. (1996) provided further evidence for the involvement of XO in inflammation. They sequenced approximately 2 kb of the 5'-flanking region and identified a variety of putative regulatory elements including an IL-6 site and potential TNF, IFN-γ, and IL-1 sites.
1.3.2. **Bone Erosion**

The presence of XO in the rheumatoid arthritic joint, and more specifically in osteoblasts, has been established and its activity is known to be increased by TNF-α, a potent resorption stimulant (Bax et al., 1992; Blake et al., 1997). Bone resorption is thought to proceed by way of a communication between TNF-α stimulated osteoblasts and osteoclasts for induction of the process, although the nature of this signal is unknown. However, H₂O₂ enhances *in vitro* bone resorption by freshly isolated rat osteoclasts in the absence of osteoblasts, and its production is increased by TNF-α *in vitro* and *in vivo*. Further evidence for XO producing the secondary messengers involved in this process is that calcium release, one of the signs of resorption, was reduced by administration of allopurinol in mouse calvaria *in vitro* (Bax et al., 1992; Blake et al., 1997).
1.4. **Other Possible Physiological Roles for Xanthine Oxidase.**

In 1982 Topham and co-workers suggested that XO plays a role in iron absorption in the small intestine and iron mobilisation in the liver. In this hypothesis, iron is absorbed, by the mucosal cells of the intestine, in the ferrous state and is oxidised to the ferric state by XO, promoting its incorporation into transferrin. XO therefore mediates the transcellular transport of iron. In the liver the D form has been shown to promote the reductive release of ferritin iron (Topham *et al.*, 1982a; 1982b).

Another radical species that is gaining increased recognition, as having a diversity of physiological and pathological functions, is nitric oxide (NO). Possible crucial regulatory roles for NO in the cardiovasculature, central nervous system, and in microbicidal activities of macrophages and neutrophils have been suggested (Moncada *et al.*, 1991). The catalysis of the production of NO from a range of nitrite and nitrate substances under anaerobic conditions by XO has recently been detailed (Millar *et al.*, 1997; Doel *et al.*, 2000; Godber *et al.*, 2000b). Specifically, nitrate substances are reduced to inorganic nitrite, which is reduced at the molybdenum centre of the enzyme, in the presence of a suitable electron donor.

Physiologically, NO is typically produced from the 5-electron oxidation of arginine to citrulline, catalysed by NO synthase. This reaction is dependent upon molecular oxygen. It has been suggested that XO could act in a complementary fashion to NO synthase, producing NO in ischaemic conditions when NO synthase activity is diminished (Zhang *et al.*, 1997). Further to this, the formation of peroxynitrite (ONOO-), another highly reactive species implicated in a number of (patho)physiological situations, from the rapid reaction between NO and superoxide, catalysed by XO, has recently been detailed (Godber *et al.*, 2000a).
The recent accumulation of evidence that XO has the ability to produce NO has generated some interesting hypotheses of the physiological role of the enzyme. It is known that XO is located both intra- as well as extracellularly (Rouquette \textit{et al.}, 1998), particularly in epithelial cells. Enzyme of high activity is especially found in the gut endothelium. Here, the extracellular enzyme provides further functions. It has been suggested that XO acts to produce vasodilatory NO from nitrate and nitrite substances in the endothelium (Millar \textit{et al.}, 1997; Doel \textit{et al.}, 2000; Godber \textit{et al.}, 2000b). That NO synthase requires oxygen to produce NO and that XO produces higher quantities of NO in the absence of oxygen suggests a possible complementary role of XO to NO synthase in endothelial NO production. Furthermore, XO bound to the outside of epithelial gut cells has been ascribed a role in bacteriostasis in the gut (Godber \textit{et al.}, 2000a). An interesting note is the presence of XO in high quantities in mammalian milk and the marked increase in XO activity \textit{postpartum} (Brown \textit{et al.}, 1995). It has been postulated that the XO in milk acts as a bacteriostatic agent, producing NO from nitrite, where the neonatal gut is immature in terms of gut flora (Stevens \textit{et al.}, 2000).

Krenitsky and his co-workers proposed another protective role for XO in the detoxification of xenobiotics (Krenitsky \textit{et al.} 1974; 1986). It was suggested that, along with aldehyde oxidase, XO provides an effective "biochemical barrier" to potentially hazardous substituted pyrimidines in nature (Krenitsky \textit{et al.}, 1972). A salutary example of this may be the oxidative activation of the prodrug 6-deoxyacyclovir (Krenitsky \textit{et al.}, 1984). Evidence exists of the conversion of this to the anti-herpetic agent acyclovir after oral administration in humans. Although this compound proved an inefficient substrate \textit{in vitro}, the suggestion was made that \textit{in vivo} the extent of oxidation would be sufficient (Krenitsky \textit{et al.}, 1986).
1.5. DISTRIBUTION AND LOCALISATION OF HXO.

Xanthine oxidase is known to be widely distributed in nature (Krenitsky et al., 1974). Enzymes from a wide variety of sources are essentially the same in terms of molecular weight and molybdenum, iron and flavin content, whilst also displaying similar substrate specificities. Although the observed electron acceptor specificities differ markedly, the suggestion has been made that the enzyme possibly evolved from a common primitive enzyme (Krenitsky et al., 1974).

Jarasch and co-workers (1981; 1986) showed the enzyme to be present in virtually all tissues in some species. Using immunolocalisation techniques, XO was found to be confined to the cytoplasm of capillary endothelial cells in tissues including mammary gland, liver, lung, intestine and heart. The enzyme was also detected in the epithelial cells of the mammary gland (Jarasch et al., 1981; 1986).

The confusion and conjecture surrounding the primary role of XO in tissue has led to studies attempting to locate the presence and activity of the enzyme in human sources. Discussed here are the findings of research on tissues particularly relevant to this investigation, namely the liver and the heart.
1.5.1. **The Liver.**

In 1974, Krenitsky *et al.* reported the detection of XO activity in the liver and small intestine of all animals. However, until recently there was very little information available regarding human XO.

In 1986 Krenitsky *et al.* purified XO 2000-fold from post-mortem human liver. The last step of the procedure involved affinity chromatography using a guanine analogue. The average particle mass of the enzyme was 300 kDa as determined by size-exclusion chromatography. This, together with results of gel electrophoresis under denaturing conditions, suggested that the native enzyme was composed of two subunits of approximately 150 kDa each. The electrophoretic patterns also indicated that a portion of these subunits had undergone partial proteolysis, yielding additional bands at 135, 95, 55 & 38 kDa. In a direct comparison with XO from bovine milk, the human liver enzyme showed a similar specificity toward purine substrates, with a high (1800 nmol/min/mg) specific activity toward xanthine.

Moriwaki *et al.* (1993) purified XO 1600-fold from human cadaver liver cytosol by an affinity technique. The purified enzyme was shown as a single band of 300 kDa on native gels and 150 kDa on SDS-PAGE, suggesting homogeneity. They used this purified enzyme to raise a polyclonal antibody against XO in rabbits. Immunostaining of frozen human hepatic tissue sections showed that the cytoplasm of hepatocytes and endothelial lining cells were stained. In a number of other tissues, the XO antigen was detected only in the endothelial lining cells from heart, kidney, brain, aorta, lung and mesentery, except for the duodenal mucousa cells.

Sarnesto *et al.* (1996) raised a polyclonal antibody to human milk and used this to identify XO in human tissues. Human liver was obtained during transplantation and deep frozen in liquid nitrogen prior to storage at -70 °C. Western blotting of liver homogenates identified
bands at 143, 125 and 87 kDa to be XO protein. By use of ELISA, XO concentrations in tissue homogenates were found to be 146 ± 70 ng/mg total protein in liver and 556 ± 320 in intestine. The authors also obtained high specific activities of 2.7-3.0 μmol/min/mg for these tissues, concluding that the amount of inactive XO in liver and intestine is small.

Previous immunoaffinity purifications of human liver XO have been effected by Hellsten-Westing (1993), Saksela and colleagues (1999) and Choudhury (2001). A monoclonal antibody to human milk XO was generated by Hellsten-Westing (1993). Immunoaffinity purification of XO from human post-mortem liver yielded fragmented enzyme of 155, 143 and 95 kDa, as determined by SDS-PAGE. Saksela et al. (1999) used a polyclonal antibody to immunoaffinity purify XO from donor livers, yielding fragments of 130 and 85 kDa, as well as the 150 kDa polypeptide. No values regarding the specific activities of the liver XO purified in either of these studies were given. Interestingly, however, this latter group did report that if the integrity of the mitochondria is ensured during homogenisation of liver tissue, almost all XO activity could be preserved in the D form, suggesting O activity to be an artefact of tissue preparation. Recently, Choudhury (2001) reported immunoaffinity purified XO to have lower specific activity than previously suggested (200 nmol/min/mg). It has been suggested that only high activity XO will bind to the affinity matrix used by Krenitsky et al. (1986), thereby yielding a specific activity not truly representative of the in vivo enzyme (Harrison, 2002). However, this does not account for the discrepancy between the specific activities for liver XO reported by Sarnesto et al. (1996) and Choudhury (2001), respectively.
1.5.2. **The Heart.**

If XO does play a key role in I/R damage, then a primary organ for investigation is obviously the heart, given the scope for possible myocardial infarction produced by a ROS-generating enzyme. However, reports of XO in human heart are, at best, confusing. In 1989 Wajner and Harkness, using postmortem heart and high (0.75 mM) xanthine concentrations in a spectrophotometric assay, reported total XO activity in excess of that found in liver and 5,000 times the activity reported by Muxfeldt and Schaper (1987). However, this appears to be an anomalous result as, in general, heart XO activity has been shown to be absent or low in humans, while immunohistochemical data are more positive (Table 1.1).

In 1993, Abadeh and co-workers isolated xanthine oxidase from human heart by immunoaffinity chromatography, and showed that it has low specific activity, comparable to that found in human milk. They suggest that the enzyme may be present primarily in 'inactive' forms in human heart, as in milk (Abadeh et al., 1993). This would explain the failure of previous groups to identify its presence in human heart, and advises against the utilisation of methods relying solely on detection of the products of its activity. To this end, the researchers utilising immunoaffinity techniques (Abadeh et al., 1992; Hellsten-Westling, 1993; Moriwaki et al., 1993) generally experienced success when attempting to detect XO in human heart, the only exception being the group of Linder et al. (1999). It is worth noting that, although unreactive toward traditional purine substrates, the enzyme would be capable of generating free radicals from NADH and therefore may still play a role in the ROS mediated tissue damage in I/R injury.
### Table 1.1 The results of attempts to detect XO activity in human heart

Except where indicated, 1 unit (U) = formation of 1μmol urate/min.

<table>
<thead>
<tr>
<th>Author</th>
<th>Technique used for detection.</th>
<th>Detection of XO (Total XO activity [mU/g tissue wet weight] quoted where applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Muxfeldt and Schaper, 1987)</td>
<td>HPLC. Homogenate, room temp.</td>
<td>Positive (1.3)</td>
</tr>
<tr>
<td>(de Jong et al., 1990)</td>
<td>HPLC. Isolated, perfused heart, 37°C.</td>
<td>Positive (0.3)</td>
</tr>
<tr>
<td>(Podzuweit et al., 1991)</td>
<td>HPLC. Homogenate, 25°C.</td>
<td>Negative</td>
</tr>
<tr>
<td>(Eddy et al., 1987)</td>
<td>Spectrophotometric and fluorimetric. Homogenate, (temp. not stated).</td>
<td>Negative</td>
</tr>
<tr>
<td>(Wajner and Harkness, 1989)</td>
<td>Spectrophotometric. Homogenate, 25°C.</td>
<td>Positive (1.2 mU/mg protein i.e. approx. 6 U/g tissue)</td>
</tr>
<tr>
<td>(Grum et al., 1989)</td>
<td>Radiochemical. Homogenate, 37°C.</td>
<td>Negative</td>
</tr>
<tr>
<td>(Kooij et al., 1992)</td>
<td>Histochemical.</td>
<td>Negative</td>
</tr>
<tr>
<td>(Abadeh et al., 1993)</td>
<td>Immunooaffinity purification Homogenate, 4°C.</td>
<td>Positive</td>
</tr>
<tr>
<td>(Hellsten-Westing, 1993)</td>
<td>Immunohistochemical.</td>
<td>Positive (vascular smooth muscle cells, capillary endothelial cells, small venules and arterioles)</td>
</tr>
<tr>
<td>(Moriwaki et al., 1993)</td>
<td>Immunohistochemical.</td>
<td>Positive (endothelial cells)</td>
</tr>
<tr>
<td>(Linder et al., 1999)</td>
<td>Immunohistochemical.</td>
<td>Negative</td>
</tr>
</tbody>
</table>
The apparent low detectability of xanthine oxidase in human heart tissue, in comparison to liver, leads to the thoughts that either the enzyme is in very low amounts in human heart tissue or, intriguingly, that the enzyme displays a tissue specificity with regard to its activity. This second suggestion further complicates the issue of a physiological role for the enzyme, whilst also leading to speculation of its tissue-specific post-translational activation (Harrison, 1997).

In other mammals, the presence of the enzyme in heart has been more conclusively determined. Rat cardiac XO, for example, has been detected by several groups in either whole isolated perfused (Schoutsen et al., 1983; de Jong et al., 1990) or homogenised heart (Grum et al., 1989; Kaminski and Jezewska, 1979; Schoutsen et al., 1983; Muxfeldt and Schaper, 1987; Eddy et al., 1987). The activity was such that traditional assay methods, i.e. spectrophotometric and fluorimetric, were able to detect the activity. XO activity ranged from 10 to 77 mU/g wet weight (Schoutsen et al., 1983 and Eddy et al., 1987, respectively).

In comparison, rabbit heart enzyme has proved to have much lower activity ((Muxfeldt and Schaper, 1987; Schoutsen and de Jong, 1987; Wajner and Harkness, 1989; de Jong et al., 1990) with some groups unable to detect XO activity (Grum et al., 1989; Downey et al., 1987). However, Wajner and Harkness (1989) suggested that this apparent low activity might be due to the presence of a high molecular weight cytosolic inhibitor, again claiming to overcome its effects by the use of high substrate concentrations.
CHAPTER 1: INTRODUCTION

1.6. ANTIBODIES TO XO.

Naturally occurring antibodies to XO (XOab) have been detected in the serum of "normal" subjects. The immunogen was initially thought to be ingested bovine enzyme. Whilst absorption of intact and enzymically active enzyme from the intestine is contentious (Ho and Clifford, 1976; McCarthy and Long, 1976; Clark et al., 1976; Volp and Lage, 1977; Zikakis et al., 1977), it is feasible that circulating XOab may have arisen in response to proteolytic fragments of bovine enzyme. However, two studies (Lewis and Ng, 1991; Benboubetra et al., 1997) suggest otherwise. Lewis and Ng (1991) discovered there to be no significant difference in the level of XO antibodies between European and Chinese subjects, despite a large disparity in bovine milk product consumption between the two groups.

Bruder and co-workers used bovine milk XO as antigen when determining the level of XOab in human serum. They found that IgG was the predominant class raised to the bovine XO (Bruder et al., 1984). When human XO was used in a similar study (Benboubetra et al., 1997), IgM XOab were found to be present at levels markedly higher than those of IgG. The level of IgM XOab in "normal" human serum represents approximately 3% of the total IgM class of immunoglobulin and the immunogen was shown to be human enzyme. It had previously been shown that heart disease patients demonstrated elevated levels of IgM XOab (Harrison et al., 1990). Higher XOab levels in young, premenopausal females are likely to be in response to hormonal, rather than dietary differences, although evidence supporting this theory is limited (Lewis and Ng, 1991).

The source of human XO leading to this apparent auto-immunisation is unclear, although it may be released from capillary endothelium due to normal or traumatic cell turnover (Bruder et al., 1984; Jarasch et al., 1986; Lewis and Ng, 1991). The reported concentrations of circulating XO in healthy human subjects range widely, from 0-4200 mU/l
CHAPTER 1: INTRODUCTION

(1mU = 1nmol urate or isoxanthopterin/min) (Martí et al., 2001; Harrison, 2002). The consensus is that serum concentration in normal healthy individuals is generally very low, with an estimated specific activity of approximately 100 nmol/min/mg being stated by Harrison (2002). However, increased levels of circulating XO have been demonstrated in some disease states, especially in the early acute phase of viral hepatitis. Elevated levels of circulating XO have also shown to occur in rheumatoid arthritis, mixed connective tissue disease, scleroderma and atherosclerosis (Harrison, 2002). Increased plasma levels of XO following human liver and intestine ischaemia-reperfusion have also been demonstrated (Tan et al., 1995; Pesonen et al., 1998).

It is likely that circulating XO may not be detected solely on the basis of conventional xanthine oxidase activity, especially in the presence of high concentrations of XOab (Jarasch et al., 1986). Immunoprecipitation experiments identified XO as the source of NADH oxidase activity in human plasma, which, as discussed earlier, is also linked with ROS production (Blake et al., 1997). Also, immune complexes of XO of both IgM and IgG isotypes have been detected in circulation in normal (Ng and Lewis, 1994) the immune complexes existing in an excess of free XOab. Therefore, this autoantibody appears to be performing a functional role, eliminating the potentially hazardous enzyme from the circulation. It is known that preeclampsia in pregnant women leads to hyperuricemia and endothelial dysfunction, XO being a possible candidate in the pathogenesis of this condition. Many et al. (1996) found no correlation between preeclampsia and the serum concentration of IgM specific to XO, or in the relative amount of IgM class XOab to total IgM. However, this may reflect the use of non-human XO in the ELISA to detect IgM XOab (Many et al., 1996).

The ability of the body to distinguish between "self" and "non-self" is a cardinal feature of the specificity of the acquired immune system. Therefore it would appear that in the case of the specific anti-human XOab, this ability has been partially compromised, resulting in an autoimmune response. However, there seems to be no detrimental systemic effect
produced by the presence of the specific XOab. Evidence does exist for an immune response to be mounted in the case of an entity, be it "foreign" or "self", if it exhibits a "danger" to the individual (Matzinger, 1994). By the same token, tolerance may be afforded to a foreign entity if it poses no danger. The basis of the so-called "danger hypothesis" is that an immune response will be produced if the entity causes damage. Therefore, the auto-immune response to HXO may be as a direct consequence of circulating enzyme producing damaging oxygen metabolites.
1.7. AIMS.

As discussed, information regarding the activity and function of XO from human sources is limited. Human milk enzyme is readily available, easily purified and currently the subject of extensive research. However, extrapolation of data obtained from this enzyme to human XO as a whole is not possible given the discrepancy in activity between the milk and liver forms.

The aim of this study is to purify and compare properties of XO from different human tissue, namely the liver and the heart. The apparent low activity of human heart enzyme suggests purification relying on the operability of the molybdenum active site, i.e. that at which traditional reducing substrates interact, would appear futile. Moreover, the success attained by detection of the enzyme in cardiac tissue by several groups using antibodies suggests the use of an immunoaffinity approach. This method is both specific for the protein, whilst also being unable to distinguish between active and inactive forms of the enzyme, therefore alluding to the "true" state of the *in vivo* enzyme. Therefore, the working hypothesis in this study is that if human heart does contain XO, then it is present in small amounts or is of low activity compared to liver, or is a combination of these two determinants.

Anti-(XO) polyclonal antibodies are to be generated and purified. These will then be used as the tool for purification of XO via immunoaffinity chromatography. Once the conditions for purification are standardised, attempts will be made to purify XO from human tissue, namely the liver and heart. Given the confusion existing as to the enzymes primary role, this study ultimately intends to aid in the elucidation of the function of XO.
Chapter 2

General materials and methods
2. General Materials and Methods

2.1. Materials

2.1.1. Chemicals

Unless otherwise stated all chemicals were obtained from Sigma, Poole, Dorset; BDH, Poole, Dorset; or Fisons, Loughborough, Leicestershire.

2.1.2. Column Chromatography Matrices

CNBr-Activated Sepharose 4B gel was obtained from Pharmacia Biotech, Uppsala, Sweden. Heparin-agarose was obtained from Sigma.

2.1.3. Instruments

Centrifugation was performed in a Sorvall RC-5B Refrigerated Superpeed Centrifuge (DuPont instruments) or in an MSE Centaur 2 benchtop centrifuge. Microcentrifugation was carried out in a MSE Microcentaur.

Immunoaffinity and heparin chromatography was monitored at 280 nm using an LKB Uvicord Type 4701A connected to a Rikandenki Chart Recorder. Fluorescent assays were carried out on a Perkin Elmer LS-5B Luminescence Spectrometer.

Absorbance Spectra were obtained from a Cecil CE 6600 Multimode Computing UV Spectrophotometer, or a Cary 100 Bio UV-Visible Spectrophotometer.

SDS-PAGE was performed using a Bio-Rad Mini Protein II system.
2.1.4. ANTIBODIES

Horseradish peroxidase conjugated anti-human, rabbit IgG and anti-rabbit antibodies were obtained from Sigma Immunochemicals.
2.2. METHODS

2.2.1. PROTEIN ESTIMATION

Protein estimations were carried out using the dye-binding method of Bradford (1976). Bovine serum albumin (BSA) at a concentration of 0.1 mg/ml was used as stock solution. A standard curve was obtained using 20-80 μl of stock BSA solution diluted to a volume of 100 μl. 0.9 ml of Bio-Rad protein assay dye reagent (Bio-Rad, Hemel Hempstead, Hertfordshire) was added to each sample, mixed and left to develop for 10 min. The $A_{595}$ was then measured using Cecil CE 6600 Multimode Computing UV Spectrophotometer. Comparisons using rabbit IgG and HXO were performed (Figure 2.1). A conversion factor of x2 for rabbit IgG was required when assaying this protein against a BSA standard curve.

![Graph](image)

Figure 2.1. Protein assay standard curves obtained with BSA, HXO and rabbit IgG ($n=3$).
2.2.2. **Xanthine Oxidase Activity Assays**

2.2.2.1. **Xanthine Assay for XO Activity (Avis et al., 1956).**

Total XO activity was assayed by following the rate of urate production in air-saturated 50 mM Na/Bicine buffer, pH 8.3 (containing 50 mM NaCl), at 25°C with 0.1 mM xanthine and 0.5 mM NAD\(^+\), spectrophotometrically at 295 nm. For the measurement of oxidase activity, 0.5 mM NAD\(^+\) was omitted from the assay. The extinction coefficient for urate is 9.6 x10\(^3\) M\(^{-1}\) cm\(^{-1}\).

The O form of XO uses oxygen efficiently under turnover conditions but has negligible reactivity with NAD\(^+\). It should be noted that although D form prefers NAD\(^+\) to O\(_2\) as oxidising substrate, it is able to use the latter in the course of turnover, albeit with much lower activity than the O form (Waud and Rajagopalan, 1976; Saito and Nishino, 1989; Hille and Nishino, 1995). However, it has become accepted practice to determine O form activity as stated above (Waud and Rajagopalan, 1976; Saito and Nishino, 1989; Abadeh et al., 1992; Sanders et al., 1997) and, for continuity, that convention was adopted in this study.

2.2.2.2. **NADH Oxidase Activity**

Prior to assaying XO for NADH oxidase activity, dehydrogenase form of the enzyme was obtained by incubation of purified enzyme in 50 mM Na/Bicine pH 8.3, containing 10 mM DTT, at 37°C for 40 min. Dithiothreitol was removed by gel filtration on Sephadex G25. The dehydrogenase form obtained by this procedure was routinely 90% as determined previously (section 2.2.2.1) Sanders et al. (1997).

With 0.1 mM NADH as a substrate, NADH utilisation in air-saturated 50 mM sodium phosphate pH 7.4 at 25°C, was followed spectrophotometrically at 340 nm. The extinction coefficient for NADH is 6.22 mM\(^{-1}\) cm\(^{-1}\).
2.2.2.3. PTERIN ASSAY FOR XO ACTIVITY (BECKMAN ET AL., 1989)

To measure XO in samples with low activity, for example human milk, the fluorescence assay of Beckman et al. (1989) was used. This measures the XO-catalysed conversion of pterin (2-amino-4-hydroxy pteridine) to the fluorescent product isoxanthopterin, and total dehydrogenase and oxidase activity is determined.

Assays were performed at room temperature. The excitation and emission wavelengths were set at 345 and 390 nm respectively; the slit width was 5 nm. For a typical assay 20 μl of sample was diluted to a volume of 960 μl in the cuvette using 50 mM potassium phosphate buffer, pH 7.4 (containing 0.1 mM EDTA). The fluorescence was then monitored to obtain a baseline. Pterin was added to a final concentration of 10 μM, along with the same concentration of methylene blue. The increase in fluorescence was then recorded over several minutes to obtain total (oxidase + dehydrogenase) activity. The reaction was then stopped by the addition of 50 μM allopurinol. The assay was calibrated by successive additions of 4 pmoles isoxanthopterin.
2.2.3. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

SDS-PAGE was performed following the method of Laemmli (1970). The separating gel consisted of 10% (w/v) acrylamide solution [9.75% (w/v) acrylamide, 0.25% (w/v) N,N'-methylene bisacrylamide (Flowgen, Sittingbome, Kent)], in 0.375 M Tris-HCl, pH 8.8 with 0.1% (w/v) SDS. Polymerisation was initiated by the addition of 0.1% (w/v) ammonium persulphate and 0.4 µl/ml Temed. Samples, typically containing 1-10 µg protein for pure samples, 100 µg for protein mixtures, were mixed with sample buffer (62.4 mM Tris-HCl, pH 6.8; 1.0% (w/v) SDS; 50% (v/v) glycerol; 10% (v/v) 2 mercaptoethanol; 0.005% (w/v) bromophenol blue) in a ratio of 1:1. They were then boiled for 90 sec before being loaded onto the stacking gel (5% (w/v) acrylamide solution [4.876% (w/v) acrylamide, 0.124% (w/v)N,N'-methylene bisacrylamide], in 0.125 M Tris-HCl, pH 6.80 with 1% SDS; polymerisation was initiated as above).

Gels were electrophoresed at 70 mV until the samples had reached the separating gel, then at 200 V, in running buffer (25 mM Tris-HCl, pH 8.3; 0.192 M glycine; 0.1% (w/v) SDS). Gels were then fixed and stained in 0.1% (w/v) Coomasie Brilliant Blue reagent in 45% (v/v) methanol; 10% (v/v) acetic acid, unless used for blotting. Destaining was performed using 50% (v/v) methanol; 10% (v/v) acetic acid. Molecular weights of proteins contained in each sample were estimated by comparing their Rm (Relative Electrophoretic Mobility) to those of protein standards.
2.2.4. Western Blotting

2.2.4.1. Electrophoresis

A nitrocellulose membrane and filter papers (18) were cut to the same size as the SDS-PAGE gel and were then soaked in transfer buffer (39 mM glycine; 48 mM tris; 1.3 mM SDS; 20% (v/v) methanol). Filter papers (9), nitrocellulose membrane and gel were then laid on the anode of a NovaBlot Unit (Pharmacia), carefully avoiding the formation of air bubbles. Once the cathode was in position, electrophoresis was carried out at constant current (0.8 mA/cm² gel).

The time for transfer was 1.5 h, after which the gel was stained with Coomasie Brilliant Blue Reagent to ensure successful transfer of the protein to the membrane. The nitrocellulose membrane was also stained with Ponceau S for band visualisation. The position of each band was marked on the membrane prior to washing for 5 min in distilled water, then TBS (10 mM Tris, 0.9% NaCl, in 1L distilled H₂O, pH 7.4). The membrane was then left to block overnight in 2% (w/v) BSA in PBS (phosphate buffered saline; 24 mM Na₂HPO₄, 5.88 mM KH₂PO₄, 120 mM NaCl).

2.2.4.2. Immunoblotting with Anti-(XO)-Antibodies

The membrane was first washed in TBS, containing 0.05% (v/v) Tween 20 (TBST). The primary antibody (immunoaffinity purified rabbit anti-HXO-antibodies or rabbit anti-HXO-serum) was diluted 1:1000 times in TBST, containing 1% BSA, applied to the blot and incubated at 37°C for 1.5 h. The membrane was then washed three times with TBST (5 min/wash). The secondary antibody (anti-rabbit-IgG peroxidase conjugate) was diluted 1:2000 times and applied to the blot (1 h, room temp.).
The membrane was washed a further three times with TBST and once with TBS before adding the visualisation substrate (Peroxidase substrate was 4-chloro-1-naphthol in 3 ml/ml methanol, stored at -20°C (CN solution); the working substrate was 2 ml CN solution in 10 ml TBS containing 10 μl H₂O₂). The bands were allowed to develop for up to 1 h at room temperature, after which the membrane was washed in TBS.
2.2.5. **Statistical Analysis**

Experimental statistical variation was expressed in one of the following two ways:

2.2.5.1. **Standard Deviation**

Standard deviation (SD) is a measure of the spread or dispersion of a set of data. It is calculated by taking the square root of the variance. SD may be calculated as in Equation 2.1, where \( n \) is the number of arguments, \( x \) is an individual data point and \( x\text{-bar} \) is the mean. Unless otherwise stated, the standard deviation was determined for all protein and activity assays.

![Equation 2.1 For calculating Standard Deviation.](image)

2.2.5.2. **Coefficient of Variation**

The coefficient of variation (CV) measures the spread of a set of data as a proportion of its mean. It is the ratio of the sample standard deviation to the sample mean and is expressed as a percentage (Equation 2.2). A coefficient of variation was used to determine inter-assay variation when determining titre levels of antisera, or measuring protein content by ELISA (Section 4.3.1.2).
Equation 2.2 For calculating Coefficient of Variation.

\[ \text{CV} = \left( \frac{SD}{\bar{X}} \right) \times 100 \]
Chapter 3

Xanthine oxidase from human milk: purification and stability
3. **Xanthine Oxidase from Human Milk:**

**Purification and Stability**

3.1. **Introduction**

Purified human XO was needed throughout this investigation for several purposes, including generation and purification of anti-XO antibodies and in order to determine the optimum conditions for purification of XO from human tissue. Bovine milk XO has been studied for many years largely due to its availability and relative ease of purification. However, effective purification of the human milk enzyme has only recently been reported (Abadeh et al., 1992).

Research into milk xanthine oxidase can be traced back to (Schardinger, 1902) and has largely been confined to bovine samples. However, it was not until 1924 that a partially purified form of the milk enzyme was obtained (Dixon and Thurlow, 1924). It was shown to utilise hypoxanthine, xanthine and aldehydes as reducing substrates; the first evidence that XO was indeed the Schardinger enzyme.

Utilising mostly pure XO, the presence of flavin and another chromophore was uncovered in separate studies by Ball (1939) and Corran et al. (1939). In 1953 Green and Beinert, and also Totter et al., identified molybdenum as a constituent of purified XO following nutritional studies. Subsequently, the effect of supplemental iron on XO activity in rat liver was investigated by Richert and Westerfield (1954), who determined iron, FAD and molybdenum to be present in a 8:2:1 ratio. These authors also proposed the non-flavin chromophore to be iron.
Avis and colleagues (1956) developed a procedure that yielded crystalline enzyme from milk. The protein (A₂₈₀) to flavin (A₄₅₀) ratio (PFR) of 5.0-5.2 was obtained for the XO in this study and is still regarded as that required of pure enzyme. In addition, sedimentation and diffusion data led to a molecular weight of 290 kDa (Avis et al., 1955).

Early attempts at purification tended to be harsh by modern standards, involving treatment with solvents, acid, proteases and heat, as well as drying (Massey and Harris, 1997). Proteases, e.g. trypsin, were included in order to remove casein and release the enzyme from the milk fat globule membrane (MFGM) (Briley and Eisenthal, 1974). However, this resulted in masking the occurrence of the D form of the enzyme, now believed to contribute as much as 90% in fresh milk XO (Harrison, 1997).

Among the column chromatography methods used during purification, calcium phosphate gel has been the most popular. However, this has been unreliable for the purification of HXO in our laboratory. Folate, a competitive inhibitor of XO, has also been used to purify HXO (Nishino and Tamura, 1991; Ventom et al., 1988). This method yields high activity enzyme, as demolybdo XO will not bind the ligand. Naturally, as human milk XO is mostly in this form, folate chromatography is not suitable for total retrieval of human enzyme from crude human milk extract.

Milk contains a suspension of discrete triglyceride micelles encapsulated by a fat globule membrane. The MFGM is derived from the apical plasma membrane of mammary epithelial cells and consists of three layers: an inner proteinaceous coat, a unit membrane and a glycocalyx (Valivullah and Keenan, 1989; Welsch et al., 1988). The protein layer contains two major proteins, xanthine oxidase and butyrophilin, a 67kDa acidic glycoprotein (Buchheim et al., 1988). Bovine butyrophilin is known to have a single membrane-spanning region, the cytoplasmic C-terminus being responsible for anchoring XO to the MFGM (Valivullah and Keenan, 1989; Ishii et al., 1995).
Purification of the human milk enzyme has been optimised in our laboratory in recent years (Abadeh et al., 1992; Sanders et al., 1997). Based upon initial research by Nakamura and Yamazaki (1982), the first stage in purification involves the partitioning of the cream, rather than attempting purification from whole milk as used in another study by Hunt and Massey, 1992. Following removal of the XO from the MFGM, further purification exploits the enzyme's affinity for heparin, a heterogeneous polyanionic glycosaminoglycan (Sanders et al., 1997). Adachi and colleagues (1993) first demonstrated the affinity of purified human milk XO for heparin-Sepharose. The mechanism of binding is believed to be electrostatic in nature, involving separate lysine and arginine rich regions of XO. These regions occur within the molybdenum domain of the enzyme, although heparin binding is not thought to inhibit enzyme activity (Fukushima et al., 1995).

The PFR obtained for milk HXO is the same as expected for pure cow enzyme. This method is still preferred when determining purity, especially given the unpredictability of XO activity in human milk post-partum (Brown et al., 1995).

Approximately 5mg of pure enzyme were obtained from 1 litre of frozen human milk using the procedure outlined by Sanders et al. (1997). Recent improvements, namely inclusion of DTT in all buffers, and immediate centrifugation on thawing, have increased the HXO obtained by around 4 fold (Godber, 1998). Both alterations optimise the removal of HXO from the MFGM. Another change has been the omission of salicylate (Hart et al., 1970).

This chapter describes the purification of human milk XO. Also investigated are the effects that possible immunoaffinity eluants will have on HXO.
3.2. METHODS

3.2.1. PREPARATION OF EXTRACT OF XO FROM HUMAN MILK

The method for obtaining an extract of XO was a modified version of that of Nakamura and Yamazaki (1982). Mothers in the Bath and Chippenham area supplied fresh milk. Frozen milk was supplied by local hospitals and stored in 20-100 ml quantities at -20°C for up to 8 weeks. Fresh and frozen samples were treated identically following thawing of the frozen milk.

The milk was centrifuged (3,000 g, 6°C, 30 min) and the upper (cream) layer was collected. The cream was washed with 5 volumes 0.2 M K$_2$HPO$_4$ (containing 1 mM EDTA and 2.5 mM DTT) and stirred for 2 h before being centrifuged (3,000 g, 6°C, 30 min). The subnatant was stirred with 15% (v/v) butanol and 15% (v/v) ammonium sulphate (1 h, 4°C). The mixture was then centrifuged (13,000 g, 20 min), the solid precipitate discarded and the subnatant stirred with an additional 20% (v/v) ammonium sulphate (45 min, 4°C). The XO preparation was obtained as a precipitate following centrifugation (10,000 g, 30 min) and resuspended in "heparin buffer" (25 mM sodium phosphate buffer, pH 7.5, containing 1 mM EDTA). The suspension was then dialysed overnight against the same buffer in order to remove excess ammonium sulphate. The following day, fine particles were removed from the suspension by centrifugation (26,700 g, 1 h) and filtering through a 0.45 μm filter.

3.2.2. HEPARIN CHROMATOGRAPHY

A 10 ml column of heparin bound to 4% crosslinked agarose was washed before use with heparin buffer. The crude enzyme was applied slowly to the column. The unbound impurities were removed from the column with heparin buffer, then with the same buffer containing 0.08 M NaCl until the $A_{280}$ of the eluant reached a constant baseline. The
enzyme was eluted with heparin buffer containing 0.4 M NaCl, and dialysed against 20 mM Na-Bicine buffer, pH 8.3 (containing 50 mM NaCl). The heparin column was washed with heparin buffer containing 1.0 M NaCl to remove any persisting protein on the column.

The fractions containing HXO were pooled and the purity of the enzyme assessed by SDS-PAGE and by obtaining the Protein:Flavin Ratio (PFR). Purified human milk XO was beaded into liquid nitrogen until required.

3.2.3. **PROTEIN:FLAVIN RATIO (PFR)**

Pure xanthine oxidase has a distinctive UV-visible spectrum of which the peaks at 280 nm and 450 nm indicate total protein and flavin/Fe:S centres respectively. The ratio between the two is known as the Protein:Flavin Ratio (PFR). Pure BXO has a PFR of 5, therefore, the lower the PFR, the less contamination by other (non-flavo) proteins.
3.3. RESULTS

3.3.1. PURIFICATION OF HXO FROM BREAST MILK

Human XO was routinely prepared from fresh or frozen milk (Sections 3.2.1 and 3.2.2). Typically, one litre of frozen milk would yield 15-25 mg of enzyme with a PFR of 5-5.2, as determined by UV-visible absorption spectra (Figure 3.1), following heparin chromatography (Figure 3.2). Purification was monitored at each stage by performing pterin activity assays (Table 3.1), and through SDS-PAGE (Figure 3.3).

![UV-Visible absorption spectrum of purified human milk XO.](image)

Figure 3.1 UV-Visible absorption spectrum of purified human milk XO.
Figure 3.2 Profile of human milk XO eluting from a heparin affinity column. The peak at 0.4 M NaCl corresponds to HXO removal from the column.
### Table 3.1 A typical purification of XO from 1.5 L of human milk. Activity was followed throughout purification by performing pterin assays at each step.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Total Activity (μmoles/min)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmoles/min/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>1500</td>
<td>1.54</td>
<td>23210</td>
<td>0.066</td>
<td>100</td>
</tr>
<tr>
<td>Cream</td>
<td>503</td>
<td>1.57</td>
<td>2204</td>
<td>0.712</td>
<td>102</td>
</tr>
<tr>
<td>Wash</td>
<td>476</td>
<td>2.02</td>
<td>914</td>
<td>2.21</td>
<td>131</td>
</tr>
<tr>
<td>Following 1st Amm. Sulph.</td>
<td>453</td>
<td>1.45</td>
<td>300</td>
<td>4.83</td>
<td>94</td>
</tr>
<tr>
<td>Resuspension following 2nd Amm. Sulph.</td>
<td>53</td>
<td>1.29</td>
<td>176</td>
<td>7.33</td>
<td>84</td>
</tr>
<tr>
<td>After Heparin</td>
<td>9</td>
<td>0.87</td>
<td>25.5</td>
<td>34.1</td>
<td>56</td>
</tr>
</tbody>
</table>
Figure 3.3 SDS-PAGE showing the stages in purification of human milk XO. Lanes 1 and 7 contain molecular weight markers. Lanes 2 and 3 contain milk and cream, respectively. Lanes 4 and 5 are samples following the 1st and 2nd ammonium sulphate fractionations, respectively. Lane 6 contains purified XO, following heparin chromatography.
The activity of the enzyme towards xanthine (Section 2.2.2.1) was also determined, as was the percentage oxidase in the purified HXO (Table 3.1).

<table>
<thead>
<tr>
<th>Yield X0 from human milk (mg/l)</th>
<th>PFR</th>
<th>Total activity (nmoles urate formed/min/mg)</th>
<th>% Oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.1 ± 7.3</td>
<td>5.13 ± 0.14</td>
<td>134 ± 42</td>
<td>13.4 ± 4.1</td>
</tr>
</tbody>
</table>

Table 3.2 The yields of purified human milk XO. XO activity was measured using the xanthine assay (Section 2.2.2.1). Values are means ± SD (n=7).

Brown et al. (1995) found that activity in human milk peaks around 15 days post-partum, falling thereafter by as much as 95%, which explains the variation in activities of purified milk XO. Details relating to the when the milk was obtained from the mother were not available in this study.
When purified HXO was subjected to a 7.5% SDS-PAGE, one main band with an estimated molecular weight of approximately 155 kDa was observed. The commercially available form of bovine milk XO (Biozyme Laboratories Ltd) used routinely in the laboratory is highly proteolysed in comparison to human enzyme purified in this study, containing additional bands at approximately 140, 90, 60 and 48 kDa (Figure 3.4). Degradation products occur at approximately 140, 90 and 60 kDa (Figure 3.5), if HXO is incubated at room temperature for 24h.

**Figure 3.4 SDS-PAGE of purified HXO** Lane 5 contains molecular weight markers. Lanes 1 and 3 contain 2 and 3 μg of purified milk HXO respectively. Lanes 2 and 4 contain 2 and 3 μg of commercial bovine XO (Biozyme Ltd) respectively.
Figure 3.5 SDS-PAGE showing the degradation products obtained on incubation of HXO at room temperature for 24 hours. Lanes 1 and 5 contain molecular weight markers. Lanes 2, 3 and 4 contain 2, 4 and 6 μg of HXO incubated at room temp for 24 h respectively.
3.3.2. **Assessment of the Antigenicity of Purified HXO**

The antigenicity of the purified HXO was assessed prior to its use in immunisation or antibody purification (Chapter 4) by performing an ELISA as described in Section 4.2.3. 10 μg/ml of purified HXO, commercial bovine XO (BXO, Biozyme Laboratories Ltd) or BSA was used to coat the microtitre plate (100 μl/well). Following blocking, serial dilutions of rabbit anti-(BXO) serum (provided by Dr Mustapha Benboubetra) were then added to the wells respectively and the procedure continued as in Section 4.2.3.

![Figure 3.6 ELISA assessment of the antigenicity of purified milk HXO.](image)

*Figure 3.6 ELISA assessment of the antigenicity of purified milk HXO. Separate lanes of the microtitre plate were coated with 100 μl purified HXO, BXO or BSA respectively (n=3).*

From the ELISA (Figure 3.6), it can be seen that purified milk HXO is antigenic, capable of binding rabbit anti-(BXO) antibodies. The titre of antibody able to bind to the HXO was considerably higher than that for the inert BSA, but lower than the immunogen, BXO.
3.3.3. **Effects of Eluants on HXO**

Prior to immunoaffinity purification of anti-XO antibodies, an investigation was performed to identify a suitable eluant. Overriding considerations when selecting a suitable eluant are that the protein purified, and the immunoglobulin used for this technique, could be recovered in forms as closely related to the native as possible, in terms of activity and affinity respectively. However, given that polyclonal antibodies display a wide range of affinities for the antigen, the effective removal of the bound analyte from the column is also of importance.

Eluants suitable for use with immunoadsorbents were suggested in "Affinity Chromatography: a practical approach" (Dean et al., 1985). 3 M KSCN (Abadeh et al., 1993), 4 M urea (Hellsten-Westling, 1993) and 50 mM diethylamine (Price, PhD Thesis, 1997) have been used previously when attempts have been made to immunoaffinity purify HXO. KSCN at a concentration of 3 M was used by Bruder et al. (1983) when immunoaffinity purifying XO from bovine tissue, using purified polyclonal anti-BXO antibodies. Benboubetra et al. (1997) also utilised 4 M urea when purifying anti-(XO) antibodies from serum. The choice of SDS (0.01% (w/v)) was as an alternative chaotropic reagent to urea. SDS has been used previously (at 10% (v/v)) to release IgG and HXO from protein A gels in immunoprecipitation experiments (Abadeh et al., 1993; Page et al., 1998). NaSCN (2 M) may have provided a preferred option to KSCN. Known to produce deflavo enzyme, MgCl₂ (2.8 M) represented a means of control. Anti-(XO) pAb elution using 0.1 M glycine-HCl, pH 2.5, has previously proved unsuccessful (Price, 1997), it was therefore omitted from this study.

In each of the following experiments "HXO" refers to untreated enzyme, "control" refers to enzyme incubated in PBS then gel filtered.
3.3.3.1. **INCUBATION OF HXO WITH ELUANTS**

Aliquots (0.5 mg) of purified HXO were incubated separately in one of the above eluants (Section 3.3.3) for 1 h at 4°C with mixing. To remove the eluant, each sample was gel filtered using a Sephadex G-25 (PD-10) column. As positive controls, 0.5 mg was also placed in PBS and treated identically, along with a 0.5 mg aliquot that remained completely untreated. By performing protein assays, UV-visible absorption spectra, along with pterin, xanthine and NADH oxidase activity assays, the structural and functional effects of each solution were analysed.
3.3.3.2. **Protein Assay**

The amount of protein recovered should not change during the experiment. This was verified by performing protein assays (Section 2.2.1). Whilst accepting that small differences in the assay may be due to experimental error, it is clear that 3 M KSCN and 2 M NaSCN have a dramatic effect on the enzyme, leading to anomalous results (Figure 3.7). The structural integrity of the enzyme may have been disrupted, causing it to react differently with the assay reagent used. Another possibility is that (partial) denaturation of the enzyme may have resulted in aggregation, preventing effective gel filtration of the protein. The effects of the other eluants do not appear to affect the reading.

![Graph showing protein assay results](image)

**Figure 3.7** Protein Assay performed after treating HXO with different eluants. Values are means ± SD (n=3).

In the subsequent activity assays, the amount of protein obtained for each eluant was assumed to be the same as the control.
3.3.3.3. **UV/VISIBLE SPECTRUM**

UV-visible spectra of the HXO samples were compared (Figure 3.8.a and 3.8.b). 4 M urea had little effect on HXO, the PFR obtained being similar to that of the untreated enzyme. As expected, 2.8 M MgCl₂ produced deflavo enzyme, the decrease in absorbance at 450 nm being characteristic of this form of the enzyme. This effect was also produced by 0.1% SDS (Figure 3.8.a). 3 M KSCN rendered HXO virtually unrecognisable when scanned, an effect seen to lesser extent with the 2 M NaSCN-treated enzyme and 35 mM diethylamine (Figure 3.8.b.).

![UV/visible absorption spectra of HXO when treated with different eluants](image)

*Figure 3.8.a  UV/visible absorption spectra of HXO when treated with different eluants. Effects of 4 M urea, 2.8 M MgCl₂ and 0.1% SDS, respectively, are shown.*
Figure 3.8.b  UV/visible absorption spectra of HXO when treated with different eluants. Effects of 3 M KSCN, 2 M NaSCN and 35 mM diethylamine, respectively, are shown.
3.3.3.4. **PTERIN ACTIVITIES**

The effect of the eluants on the activity toward pterin was determined by performing pterin assays as Section 2.2.2.3. (Figure 3.9). Electrons may pass either to oxygen (at the FAD site), or to methylene blue (at the Fe/S centres).

![Figure 3.9 Pterin activities of HXO treated with possible eluants (n=3). Values are means ± SD (n=3).](image)

Assuming the amount of protein present in all cases to be that of the control, the activities of the urea, NaSCN and diethylamine treated enzymes are approximately that of this untreated (control) HXO. The small increase in activity found in the 4 M urea sample may be due to error. Both the 0.1% SDS and 2.8 M MgCl₂ have a detrimental effect on the pterin assay, indicating that disruption of the enzyme at either the molybdenum site, or at the Fe:S centres has occurred in both cases.
3.3.3.5. **XANTHINE ACTIVITIES**

The effect of the eluants on the molybdenum:FAD relationship was determined by performing xanthine activity assays as in Section 2.2.2.1.

Only the enzyme treated with 4 M urea (Figure 3.10) recovered full activity. 2.8 M MgCl₂ was known to generate deflavo enzyme, thereby producing a decreased activity towards xanthine. 3 M KSCN, 2 M NaSCN, 35 mM diethylamine and 0.1% SDS all also affected the activity, showing modification had been produced at the molybdenum and/or FAD sites of the enzyme.

![Graph](image)

**Figure 3.10** Xanthine activities of HXO treated with possible eluants (*n=3*). Values are means ± SD (*n=3*).
3.3.3.6. **NADH oxidase activities**

As described earlier (Section 1.2.2) NADH uniquely donates electrons at the FAD centre. The NADH oxidase assay therefore, will determine the effects of eluants solely at this site.

![Figure 3.11](image)

*Figure 3.11 NADH oxidase activities of HXO treated with possible eluants (n=3). Values are means ± SD (n=3).*

Again, 4 M urea had least effect on the enzyme's NADH oxidase activity. Decrease in this activity suggests the removal of FAD by its hydrolysis to FMN. The trend seen with eluants apart from urea is similar to that observed for the xanthine assay, which suggests FAD disruption as a cause of the decrease in the reactivity of HXO with xanthine, with the eluants concerned.
3.4. DISCUSSION

Pure HXO was required as immunogen to produce rabbit anti-(HXO) antiserum and for affinity purification of the antiserum yielding polyclonal antibodies.

Graham et al. (1989), Hellsten-Westin et al. (1993) and Benboubetra et al. (1997) previously purified HXO for this purpose. Following salt precipitation, the latter authors used the same procedure as outlined in this study, whilst hydroxylapatite and anion exchange chromatography were utilised by Graham et al. (1989) and Hellsten-Westin et al. (1993), respectively. The activity of the milk enzyme is not of primary importance, therefore removing the need for folate chromatography, providing this enzyme is immunogenic (see Chapter 4).

As previously described, the procedure of Sanders et al. (1997) has been recently modified by Godber (1998). The omission of an initial stirring stage and inclusion of DTT in all buffers has improved the yields obtained. Reasons given for this are that stirring will fragment the MFGM, leading to release of XO into the non-cream fraction following centrifugation. The nature of binding of HXO to the MFGM is, as yet, undetermined. There is, however, increasing evidence of the association of XO with glycosaminoglycans (Adachi et al., 1993; Radi et al., 1997), found on the MFGM surface. The thiol reagent, DTT, is capable of converting reversible O form into D form. Godber (1998) argued that inclusion of DTT converts the enzyme into the D form, which has a lower heparin affinity, thereby liberating this from the MFGM.

In the present study, the reduction in salt concentration in pre-elution buffer led to a small increase in yield, without sacrificing purity. In fact, the increased volume afforded to the pre-elution stage was seen to generally result in increased purity. The anion exchange chromatography stage used by Sanders et al. (1997) is no longer required, as pure enzyme (PFR 5.0-5.2) can now be obtained post-heparin.
The antigenicity of the purified enzyme was demonstrated as it was capable of binding anti-(BXO) polyclonal antibodies, as shown by ELISA, therefore endorsing the possibility of raising and purifying polyclonal antibodies to HXO. Although the titre was not at the level seen towards commercial bovine XO, it does suggest a sharing of epitopes common to enzyme from both sources (see Chapter 4).

Elution studies were used to identify suitable conditions for recovery of pure enzyme, in as active a form as possible. The interactions that enable the formation of immune complexes are believed to include electrostatic, hydrophobic, van der Waals and hydrogen bonding forces. Therefore perturbation of all, or a majority of, these four physical interactions will need to occur for dissociation and subsequent recovery of bound ligands (Tsang and Wilkins, 1991). Because polyclonal antibodies usually bind to a number of sites on an antigen, and can therefore display high avidity, they can be difficult to elute. The harsh conditions required may therefore result in at least a partial denaturation of the antigen (Harlow and Lane, 1988). Along with recovery of active enzyme, the ability to reuse any antigen/antibody is of utmost importance. In 1991, Tsang and Wilkins identified EtMg (3.0 M MgCl₂ in 0.075 M Hepes/NaOH (pH 7.0) with 25% (v/v) ethylene glycol) as the most suitable eluant for immunoaffinity purification in an FPLC system. This eluant gave the best return in terms of total recovery (i.e. specific activity x total protein recovered). However, EtMg is highly viscous, and could not be applied to the low-pressure system used in this study. The authors also found 6.0 M urea to be one of the most effective eluants at removing bound antibody from the antigen matrix, no remaining bound material being detected post-elution.

In a previous study (Kanda et al., 1972), concentrations of urea up to 6.7 M were seen to have no affect on the xanthine activity of bovine milk or chicken liver enzyme. Accordingly, the present investigation appears to identify 4 M urea as the most suitable eluant in terms of recovered structure and function. However, urea is known to block the N-terminus of proteins (via carbamoylation), and is therefore unsuitable for elution of HXO needed for
sequencing. Sequencing of purified HXO is potentially complicated by the fact that 50-75% of eukaryotic proteins lack a free α-amino group (Dunn et al., 1993). This N-terminal modification occurs post- or co-translationally within the cell, and will be increased by urea elution.

According to Harlow and Lane (1988), of the elution conditions tested here, SDS and KSCN affect the reusability of immunoaffinity columns, and may therefore be discounted as eluants. Bruder et al. (1983) used 3 M KSCN to elute bovine tissue XO from an immunoaffinity column. Like Abadeh et al. (1993), elution proved successful. However, these researchers attributed the inability to detect XO activity in eluates to the inactivation of the enzyme by the high concentrations of this chaotropic agent. KSCN has been used previously as an alternative to CaCl₂ for FAD removal from XO (Bray, 1975) and therefore explains the reduced XO activities resulting from exposure to this eluant.

Price (1997), demonstrated the re-use of an anti-(BXO) immunoaffinity column following diethylamine facilitated elution, although no information regarding recovered enzyme activity was given. It has now been elucidated that although the UV-visible scan of diethylamine treated enzyme was poor, results from activity assays were encouraging. The pterin–methylen blue activity remained largely intact, suggesting that the integrity of the molybdenum centre was maintained. In addition, the loss in xanthine→(O₂/NAD⁺) and NADH oxidase activities may be retrievable, should the reduced activity be due to flavin removal. In conclusion, diethylamine (35 mM) has been identified as the most applicable eluants of the ones tested for the purification of XO from human tissue.
Chapter 4

Generation and purification of anti-(HXO) antibodies
4. **GENERATION AND PURIFICATION OF ANTI-(HXO) ANTIBODIES**

4.1. **INTRODUCTION**

The occurrence of endogenous anti-(XO) antibodies in human subjects is well documented (see Section 1.6). This ability of XO to elicit an immune response has been exploited, resulting in the generation of rabbit polyclonal (Graham *et al.*, 1989; Moriwaki *et al.*, 1993; Benboubetra *et al.*, 1997; Linder *et al.*, 1999) and monoclonal antibodies (Hellsten-Westin, 1993).

This chapter deals with the generation of rabbit anti-(HXO) polyclonal antibodies and their subsequent purification from serum by affinity chromatography. The use of anti-(HXO) pAbs in the detection of the enzyme is also discussed.
4.2. METHODS

4.2.1. PREPARATION OF AFFINITY COLUMNS


4.2.1.1. COUPLING OF PROTEIN TO CNBr-ACTIVATED SEPHAROSE 4B GEL

The protein to be immobilised, i.e. purified HXO (Chapter 3) or protein A, was dialysed against coupling buffer (0.1 M NaHCO₃, pH 8.3, containing 0.1 M NaCl) overnight. CNBr activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was allowed to swell in 1 mM HCl for 2 h at 4°C. During this period the gel slurry was washed three times with 1 mM HCl (250 ml/wash). The gel was then washed with coupling buffer (5 ml/ml of gel) quickly (to prevent hydrolysis of reactive groups) using a sintered glass funnel, before being mixed with the dialysed protein. This coupling suspension was incubated at 4°C for 16 h with end-over-end mixing.

The gel was then washed three times with PBS and the coupling efficiency of the gel calculated by determining the amount of protein contained in each wash. To block any remaining active sites on the beads, 0.1 M glycine (2 ml) was added to the gel and mixed for 2 h at 4°C. The gel was then washed alternately with 0.1 M sodium acetate, pH 4.0 (containing 0.5 M NaCl) and coupling buffer. This washing procedure was repeated three times to ensure that no free ligand remained ionically bound to the immobilised ligand. A column (15 ml) was packed and the gel washed thoroughly with PBS and stored in PBS containing 0.01% (v/v) sodium azide at 4°C until use.
4.2.1.2. **Ligand Coupling Efficiencies**

HXO, 20 mg, was coupled to the CNBr-activated Sepharose 4B at a ratio of 5 mg protein per ml of swollen gel. Estimation of uncoupled protein in the gel washes allowed coupling efficiencies of the enzyme to be determined. Routinely, an efficiency of coupling for HXO of 90-97% was obtained.

Protein A, 20 mg, was bound to the matrix at a ratio of 10 mg per ml swollen gel. The coupling efficiency of this ligand was 88-97%.
4.2.2. **ANTIBODY GENERATION AND PURIFICATION**

4.2.2.1. **RABBIT IMMUNISATION**

Rabbits were initially immunised with Imject Alum adjuvant (Pierce, Netherlands) (500 µl) containing 400 µg of either purified BXO (supplied by Ben Godber) or purified HXO (Sections 3.2.1. and 3.2.2.).

On day 28 a boost injection of purified BXO or purified HXO (400 µg), respectively, was administered. This represents the secondary immunogenic stimulus that produces a shift in class response, IgG antibodies appearing at higher concentrations, and with greater persistence, than IgM. Also, a maturation of the response occurs, the IgG having greater affinity for the antigen as the response develops. After 7 days, a blood sample was obtained from a peripheral ear vein and the serum anti-(XO) level assayed by ELISA (Section 4.2.3), normal (non-immunised) rabbit serum was used as the negative control.

Blood was collected from rabbits (approximately 40 ml/rabbit) displaying sufficient titre. Subsequent boosts and bleeds were continued for up to 6 months from the initial immunisation, whereupon the rabbits were sacrificed, yielding approximately 150 ml blood.

4.2.2.2. **PREPARATION OF ANTISERUM**

Collected blood was allowed to coagulate overnight at 4°C, after which time it was centrifuged at 3,000 g for 15 min. Straw-coloured serum (0.2-0.5 ml per ml blood) was aspirated off the top of the clot, aliquoted and stored at -20°C until use. Haemolysed serum (red, 0.05-0.1 ml per ml blood) was also removed from above the clot. From this sample immunoglobulin was precipitated immediately, re-dissolved and dialysed against PBS (as in Section 4.2.2.3).
4.2.2.3. Preparation of crude extract of rabbit immunoglobulins

Preparation of a crude extract of rabbit IgGs (and IgMs) from antisera by ammonium sulphate precipitation is a standard procedure performed to reduce the amount of contaminating proteins in solution prior to affinity chromatography. At 50% ammonium sulphate saturation IgGs and IgMs are precipitated from rabbit serum, whilst most other serum protein remains in solution.

The serum (up to 10 ml) was centrifuged (3,000 g, 30 min, room temp.). An equal volume of saturated ammonium sulphate solution was added (to bring to 50% saturation) and the mixture was incubated overnight at 4°C, or for 1 h at room temperature. The mixture was then centrifuged (3,000 g, 30 min, room temp.) and the supernatant discarded. The pellet was resuspended in an equal volume of PBS, pH 7.4 (containing 0.01% (w/v) sodium azide) and dialysed, or gel filtered, against the same buffer in order to remove the ammonium sulphate. The immunoglobulin preparation was then used immediately, or stored at 4°C for 2-3 weeks.

4.2.2.4. Protein A affinity purification of IgG from antisera

Total IgG can be obtained from whole serum, or an ammonium sulphate preparation (Section 4.2.2.3) by protein A affinity chromatography. Isolated from Staphylococcus aureus, protein A has a high affinity for the Fc region of IgG. Immobilisation of protein A on CNBr-activated Sepharose 4B was performed as described previously (Section 4.2.1).

Precipitated immunoglobulin (Section 4.2.2.3) was diluted 1:10 in PBS, and circulated on the column overnight at 4°C. The gel was then washed with the same buffer until the change in absorbance at 280 nm was negligible. Bound IgG was eluted with 4 M urea and dialysed overnight, at 4°C, against several changes of PBS.
4.2.2.5. **AFFINITY PURIFICATION OF RABBIT ANTI-(HXO)-ANTIBODIES**

The sample (serum precipitate or crude rabbit IgG preparation, Sections 4.2.2.3 and 4.2.2.4 respectively) was diluted 1:10 in PBS and applied to the column of bound HXO. The antisera/IgG preparations were allowed to circulate on the column overnight to allow maximum binding of anti-(HXO) antibodies to the ligand. Unbound protein was washed off the column with PBS until the change in A280 was negligible.

Following determination of their effects on HXO (Section 3.3.3), bound antibody was eluted with 4 M Urea or 35 mM diethylamine, containing 35 mM NaCl. In the case of the latter eluant, eluted fractions containing antibody were collected in tubes containing 1/5 volume 1M Na-Bicine buffer, pH 8.0, and pooled. Eluted antibody was dialysed against several changes of PBS overnight before concentrating either with PEG (Mol. Wt. 15,000-20,000) or using a 8050 stirred cell (Amicron Inc, Beverely, MA, USA; in conjunction with a XM50 Diaflo ultrafiltration membrane, Amicon).

The column was regenerated using PBS (100 ml), followed by 0.1 mM HCl (30 ml). A final wash with PBS, containing 0.01% (v/v) sodium azide was performed prior to storage at 4°C. The antibodies were analysed using ELISA (Section 4.2.3) and SDS-PAGE (Section 2.2.3) before being stored at -20°C.
4.2.3. ELISA FOR THE DETERMINATION OF ANTI-(XO) POLYCLONAL ANTIBODIES

Purified Human Xanthine Oxidase or Bovine Xanthine Oxidase (Biozyme, Bleanavon, Gwent) was suspended in coating buffer (50 mM sodium carbonate, pH 9.6) at a concentration of 10 µg/ml. The solution was added to each well (100 µl) of a flat-bottomed polystyrene microtitre plate (Labsystems, Helsinki) and the plate was incubated overnight at 4°C or for 2 h at 37°C. Wells were then washed at room temperature with 3 successive portions of PBS-(0.05-0.1%) Tween 20 (200 µl/well). Excess binding sites were blocked with PBS-(0.05-0.1%) Tween 20 containing 1% casein (1/4) (200 µl/well) for 1 1/2 h at room temperature. The wells were then washed as above. Standard serum, serum samples and antibody solutions to be assayed were diluted 100 - 51200 times in 1% (1/4) casein/PBS. They were then added in series to lanes of wells respectively and incubated overnight at 4°C or for 2 h at 37°C. After incubation, the plate was washed as above and the secondary antibody (horseradish peroxidase conjugate), diluted 1:2000 times in 1% casein/PBS-T, was added to each well (100 µl/well). Following incubation (1 1/2 h at room temperature) the plate was washed again and staining buffer was added to each well (100 µl/well) and the colour was allowed to develop for 20-30 min. Finally, the reaction was stopped by the addition of 1 M sulphuric acid (50 µl/well) and, following 5-10 min incubation, the absorbance was read at 450 nm using a Titertek Multiskan MCC.
4.3. RESULTS

4.3.1. RABBIT ANTI-(XO) POLYCLONAL ANTIBODIES

4.3.1.1. GENERATION OF ANTIBODY

Four New Zealand White rabbits designated 195, 196, 204 and 216 were immunised with XO, as described in Section 4.2.2.1. Rabbit 195 was immunised against the bovine form of the enzyme, human XO being the immunogen for the other animals.

Five weeks after the initial immunisation, one week following the boost injection, a sample of blood was removed from each rabbit and the serum prepared (Section 4.2.2.2). The serum rabbit anti-(XO) antibody level was then determined by ELISA (Section 4.2.3), microtitre plates being coated with 10 µg/ml of purified HXO (Figure 4.1) or commercially available Bxo (Biozyme Laboratories Ltd) (Figure 4.2). Antisera dilution series were added in triplicate in order to obtain a standard curve. Titres were calculated from curves of antisera dilutions displaying good parallelism to a reference curve produced with normal rabbit serum (NRS).

With the exception of rabbit 196, the immunised rabbits produced serum of consistently high titre (Table 4.1). It would be expected that the antisera would display a higher titre of anti-(XO) antibody toward the immunogen, rather than an alternative form of the enzyme (i.e. human or bovine form of the enzyme). This was demonstrated in the case of rabbit 195, which had a titre toward its bovine XO immunogen of greater than 3.5 times that of its apparent anti-(HXO) titre.
CHAPTER 4: GENERATION AND PURIFICATION OF ANTI-(HXO) ANTIBODIES

Figure 4.1 Typical anti-(HXO) titration curves obtained from rabbit serum.

Figure 4.2 Typical anti-(BXO) titration curves obtained from rabbit serum.

In both Figures 4.1 and 4.2, serum from non-immunised rabbits, termed normal rabbit serum (NRS), was used as negative control to produce a reference curve.
Rabbits immunised against the human form of XO (204 and 216) demonstrated a higher anti-(HXO) titre than the "bovine" rabbit 195. However, both rabbit 204 and 216 exhibited an apparently higher titre toward BXO than their immunogen, HXO. This may be due to the nature of the bovine enzyme used to coat the microtitre plate. The commercial bovine enzyme used is highly proteolysed, and may have additional epitopes exposed that are exploited by immunoglobulins in the antisera, producing an apparently higher anti-(BXO) titre. This may also explain the higher titre toward the bovine enzyme observed in the normal rabbit serum, a finding in itself providing evidence of an endogenous (auto-) anti-(XO) antibody present in the serum of rabbits.

<table>
<thead>
<tr>
<th>Rabbit Antisera</th>
<th>Normal Rabbit Serum</th>
<th>195</th>
<th>196</th>
<th>204</th>
<th>216</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-immunised</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-(Human XO)</td>
<td>0.102</td>
<td>4.57</td>
<td>3.13</td>
<td>6.76</td>
<td>6.61</td>
</tr>
<tr>
<td>Titre x 10^4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-(Bovine XO)</td>
<td>0.186</td>
<td>17.0</td>
<td>1.91</td>
<td>7.55</td>
<td>7.50</td>
</tr>
<tr>
<td>Titre x 10^4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 Anti-(XO) polyclonal antibody titre obtained from Figures 4.1 and 4.2. Titre values correspond to the reciprocal of the -log_{10} dilution at 50% maximal absorbance.
4.3.1.2. **Coefficient of Variation**

A coefficient of variation (CV) for NRS anti-(HXO) titre was determined to monitor inter-assay variation when assessing titre levels of antisera (Section 2.2.5.2). Six reference curves, each from a triplicate dilution series, were obtained over a period of 1 month. The effect of freeze/thawing of the HXO, and of freshness of the buffer used to coat the plates was investigated (Figure 4.3). The anti-(HXO) titre for each reference curve was determined (Table 4.2).

**Figure 4.3 Reference curves for NRS. HXO 1 and HXO 2 had PFRs of 5.0 and 5.14 respectively (n=3). In both cases the conditions were as follows: (a) = Fresh HXO and fresh coating buffer; (b) = Defrosted HXO and fresh coating buffer; and (c) = Defrosted HXO and 1 month old coating buffer.**
Table 4.2 Titre values correspond to the reciprocal of the \(-\log_{10}\) dilution at 50\% maximal absorbance (n=3).

Although a considerable difference was seen between the two fresh enzyme preparations when using fresh buffer, overall the small difference in PFR (0.14) between the HXO samples had little effect on the titres obtained. Also, freeze/thawing of enzyme and the use of "old" buffer also appeared not to have a significant effect on the obtained titres.

Typically, enzyme used for ELISAs was thawed, with a PFR of 5-5.2. Coating buffer was stored at 4\(^\circ\)C and utilised within 1 month of preparation. It should be noted that the bacteriostatic agent sodium azide should not be used in conjunction with the ELISA if utilising a peroxidase-conjugated detection system ([Kemeny, 1991])

The mean titre for the 6 curves was determined to be 5.53 \(\times\)10\(^4\). Interassay precision was determined to have a CV of \(\pm\)20\%.

<table>
<thead>
<tr>
<th></th>
<th>Fresh Enzyme</th>
<th>Thawed Enzyme</th>
<th>Thawed Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>HXO 1 (x10(^4))</td>
<td>7.08</td>
<td>5.37</td>
<td>4.47</td>
</tr>
<tr>
<td>HXO 2 (x10(^4))</td>
<td>4.17</td>
<td>5.62</td>
<td>6.46</td>
</tr>
</tbody>
</table>
4.3.1.3. **Purification of IgG from Rabbit Serum**

In order to prolong the life and operability of the XO affinity column, whilst also ensuring as pure an antibody preparation as possible, rabbit antiserum was initially subjected to ammonium sulphate precipitation (Section 4.2.2.3). ELISAs performed (as in Section 4.2.3) detected no residual anti-XO activity in the serum following immunoglobulin precipitation. The resultant immunoglobulin preparation was gel filtered, diluted and immediately circulated slowly overnight through the HXO affinity column. It became routine, however, for IgG to be isolated by protein A chromatography (Section 4.2.2.4) prior to the HXO affinity stage (Figure 4.4). Bound protein was eluted as described previously (Section 4.2.2.5).
Figure 4.4 A 7.5% SDS-PAGE of antiserum protein purification. Lane 2 contains protein purified by protein-A chromatography. Lane 3 contains protein precipitated from rabbit serum by ammonium sulphate fractionation. Lane 4 contains whole rabbit serum. Lanes 1 and 5 contain molecular weight markers.
4.3.1.4. **PURIFICATION OF ANTI-(HXO)-IgG FROM RABBIT SERUM.**

Anti-(HXO) IgG was affinity purified from serum precipitate or crude rabbit IgG preparation (Section 4.3.1.3) using diethylamine as eluant, as described in Section 4.2.2.5 (Figure 4.5). A comparison of an anti-(HXO) antibody preparation, with and without the IgG isolation step can be seen in Figure 4.6. Purified IgG displays 2 bands when subjected to SDS-PAGE at 50 and 25 kDa, corresponding to heavy and light chains, respectively.
Figure 4.5 Elution profile of anti-(HXO) IgG from the HXO affinity column with 35 mM diethylamine used as eluant. 1 = PBS wash of unbound IgG from the column. 2 = Application of 35 mM diethylamine (containing 35 mM NaCl). 3 = Application of PBS following elution of anti-(HXO) IgG.
Figure 4.6 A 10% SDS-PAGE of affinity purified rabbit anti-(HXO) polyclonal antibodies, from whole antisera and a protein-A IgG preparation. Lane 1 and 2 contains affinity purified rabbit anti-(HXO) IgG from rabbits 195 (anti-BXO) and 204 (anti-HXO) serum, respectively, following protein-A IgG preparation. Lane 3 and 4 contains affinity purified rabbit anti-(HXO) polyclonal antibodies from rabbits 195 and 204, whole sera, respectively. Lane 5 contains molecular weight markers.

Contaminating bands at approximately 100 and 75 kDa present in the ammonium sulphate precipitated purified antibody were removed by protein A chromatography.

The yield of anti-(HXO) pAb was assessed following the recirculation of the protein-A IgG preparation through the HXO affinity column. Recirculation-elution cycles were performed until the amount of residual anti-(HXO) pAb was undetectable in the preparation by ELISA (Section 4.2.3). The yield of anti-(HXO) pAb for each antiserum is shown in Table 4.3.
CHAPTER 4: GENERATION AND PURIFICATION OF ANTI-(HXO) ANTIBODIES

Table 4.3 The yield of anti-(HXO) antibodies obtained from 10 ml of antiserum. IgG was isolated from antiserum and that specific for HXO was purified subsequently by affinity chromatography.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Elution 1 (mg)</th>
<th>Elution 2 (mg)</th>
<th>Elution 3 (mg)</th>
<th>Elution 4 (mg)</th>
<th>Total Yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>4.1</td>
<td>1.54</td>
<td>0.66</td>
<td>0.11</td>
<td>6.41</td>
</tr>
<tr>
<td>196</td>
<td>2.1</td>
<td>0.52</td>
<td>0.23</td>
<td>0</td>
<td>2.85</td>
</tr>
<tr>
<td>204</td>
<td>4.3</td>
<td>1.98</td>
<td>0.92</td>
<td>0.2</td>
<td>7.4</td>
</tr>
<tr>
<td>216</td>
<td>4.7</td>
<td>1.75</td>
<td>0.84</td>
<td>0.28</td>
<td>7.55</td>
</tr>
</tbody>
</table>

Eluted antibody from each sample of antiserum was pooled and the titre was determined from ELISA against a sample of the original antiserum (Figure 4.7). For the typical case, shown in Figure 4.7, the anti-(HXO) titre obtained for the pooled eluted antibody ($2.29 \times 10^4$) was 55% of that of the original antiserum ($4.17 \times 10^4$). Purified anti-(HXO) titres routinely ranged from approximately 50-65% of the original antiserum. Lost antibody may be of low affinity, removed from the column during the washing stage, whilst very high affinity antibody may remain bound to the HXO immobilised on the column matrix (see Section 4.3.1.5). Small losses in yield may also be attributed to adherence of antibody to membranes used in the dialysis and concentration stages of purification. It is also feasible that conditions required for elution may irreversibly disrupt antibody paratopes, which may not recover fully on dialysis. These influences will effect the final purified anti-(HXO) pAb titre.
Figure 4.7  A typical ELISA of the yield of anti-(HXO) pAb. In this instance the antiserum used was from rabbit 195, IgG prepared by protein-A affinity chromatography, and gel filtered prior to circulation on the affinity column. Antibody from 4 elution cycles was pooled and used in the ELISA.
4.3.1.5. **Efficacy of Eluant**

In order to determine the efficacy of the eluant used, following cycles of elution of antibody from the column, small quantities of gel matrix (<50 µl) were removed and subjected to SDS-PAGE (Figure 4.8). Initial elution cycles utilised 4 M urea as the eluant, however bands characteristic of IgG heavy and light chains (approximately 50 kDa and 25 kDa respectively) were present in gel samples, along with bands characteristic of HXO. Increases in concentration of urea failed to remove the contaminating bands. However, the use of 35 mM diethylamine (containing 35 mM NaCl) was found to eradicate the persisting immunoglobulin.
Figure 4.8 A 10% SDS-PAGE illustrating the removal of persisting IgG from a HXO affinity matrix. Lane 1 contains molecular weight markers. Lanes 2, 3, 4 and 5 contain extracts from an HXO affinity column subjected to urea at 4 M, 4.5 M, 5 M and 6 M respectively. Lane 6 contains an extract from the same HXO column following elution with 35 mM diethylamine (containing 35 mM NaCl).

The persistence of IgG would have most affect during subsequent initial purification attempts, the high affinity antibodies blocking sites available to IgGs of lower affinity. Other drawbacks involving the use of 4 M urea were the time required (>2 h), and high volumes required (>100 ml) for elution. The use of 35 mM diethylamine reduced the time for elution to 20 min, whilst the elution volume was reduced to approximately 40 ml. Also noteworthy is the observed degradation of the enzyme, with bands additional to the main protein band at 150 kDa, in all cases.
4.3.1.6. **PERFORMANCE OF HXO AFFINITY COLUMN**

Following the observed degradation of the immobilised enzyme after antibody purification cycles (Section 4.3.1.5), the performance of each affinity column was monitored during its use. The characteristic brown colour of xanthine oxidase was lost from the column following its first or second use with 35 mM diethylamine as the eluant. This suggests removal of the flavin moiety from the immobilised enzyme by the conditions required for elution. However, an accompanying major loss in performance of the affinity column was not experienced, suggesting the retention of antigenicity of the immobilised enzyme. A gradual decrease in performance of the column was witnessed over a period of three months (Figure 4.9).

![Graph showing yield over time](image)

*Figure 4.9 The yield obtained from 10 ml of rabbit 195 antisera. In each case IgG was prepared from the antiserum as described in Section 4.2.2.4. The yields quoted are elutions with 35 mM diethylamine (containing 35 mM NaCl) from the first circulation only, from human XO affinity column containing 20 mg total of purified HXO.*
This loss in performance may be attributed to enzyme leaking from the column matrix, or through binding anti-(HXO) pAb with very high affinity, thereby irreversibly blocking epitopes. This latter theory can be discounted as 35 mM diethylamine (containing 35 mM NaCl) was the eluant (Section 4.3.1.5). Therefore, the major reason for the loss in performance is more likely to be due to ligand leakage. Degradation of the enzyme following elution was observed on subjecting a sample of immobilised HXO to SDS-PAGE (Figure 4.8). The degradation products observed were characteristic of HXO when compared to those seen previously (Figure 3.5). However, the total amount of anti-(HXO) pAb purifiable from 10 ml of antiserum, following recirculation, fell off only marginally with time (Figure 4.9). Columns were routinely replaced after a period of 3-4 months, depending on use and performance.
4.3.1.7. **LOCALISATION OF XO IN HUMAN ENDOTHELIAL AND EPITHELIAL CELLS.**

Although it is well documented that XO is present in a wide variety of tissues, the exact subcellular localisation of XO has received little attention. Initial studies, using light and electron microscopic immunohistochemistry, indicated the enzyme to be purely cytosolic in bovine capillary endothelial cells (Jarasch *et al.*, 1981). Subsequent studies on rat hepatocytes revealed XO activity to be present in peroxisomes (Angermüller *et al.*, 1987; Dikov *et al.*, 1988). However, Ichikawa and co-workers (1992) questioned these latter findings, concluding the enzyme to be exclusively cytosolic.

The affinity purified rabbit anti-(HXO) pAb generated in this study was used as a tool by co-workers to investigate the situation in human cells (Rouquette *et al.*, 1997; Appendix 1). Confocal microscopic studies of permeabilised cultured human endothelial and epithelial cells indicated that XO was distributed diffusely throughout the cytoplasm, but with high intensity in the perinuclear region. Furthermore, the punctate staining seen in many instances is suggestive of a vesicular location. The situation in non-permeabilised cells was also intriguing. XO was found to be asymmetrically located, higher intensity of fluorescence demonstrated on the faces apposed by closely neighbouring cells. This specific distribution gives weight to the growing belief that XO is involved in ROS mediated signalling (Rouquette *et al.*, 1997; Appendix 1).
4.3.1.8. **OTHER USES OF AFFINITY PURIFIED RABBIT ANTI-(HXO) PAb.**

Once purified, the purified rabbit anti-(HXO) pAb was used for a number of purposes. Immunoprobing with the antibodies was utilised during Western blotting (Figure 4.10) whilst a sandwich ELISA developed within the laboratory utilised the antibody to detect nanogram quantities of the enzyme. Antiserum was also donated to Dr Eugene Bosmans, who developed a commercial sandwich ELISA for detection of XO (Eurogenetics, Belgium). Ultimately, however, the antibodies were to be used as a tool in order to purify the enzyme from tissue (see Chapters 5 and 6).
Figure 4.10 A 10% SDS-PAGE and corresponding Western blots demonstrating the affinity of purified anti-(HXO) IgG. Lane 1 contains purified HXO (allowed to stand at room temp for 24 hours to produce the degradation bands); lane 2 contains commercial BXO (Biozyme Laboratories Ltd) and Lane 3 contains molecular weight markers. Western blot (a) (lanes order reversed compared to SDS-PAGE) utilises whole rabbit 195 antiserum. Western blot (b) utilises purified rabbit anti-(HXO) IgG. In both cases the secondary
antibody used for detection was a goat anti-rabbit IgG (whole molecule) peroxidase conjugate.

Western blots (Figure 4.10) demonstrated that, rather than solely detecting human XO, the anti-(HXO) IgGs also showed avidity for the bovine form, indicating a degree of conservation of structure between the two enzymes. Also, the pAb was able to detect the degradation bands of HXO and BXO, indicating specificities toward separate subunits of the enzyme in the total population of purified antibody. However, the specificity of the purified pAb for XO was demonstrated, as none of the marker proteins was detected.
4.4. DISCUSSION

High titre anti-(XO) antisera were obtained routinely from rabbits immunised against the bovine (195) and human (204, 216) forms of the enzyme. Rabbit 196 produced antisera with a low titre, although it had been immunised identically to the other rabbits.

ELISA revealed that antisera displayed avidity for BXO, as well as for the immunogen, indicating a sharing of common epitopes between HXO and BXO. It would be expected that the antisera display a higher titre toward the immunogen. Although this was the case with antisera from rabbits 195, and 196 (with low titre), the opposite was true for 204 and 216, both "anti-(HXO)". This apparent greater avidity for the bovine form may be due to the exposure of a wider range of epitopes in the partially proteolysed commercial bovine enzyme used in the ELISA. These epitopes may have become exposed in the injected HXO, should the enzyme have been subject to proteolysis in vivo, but remain cloaked in the predominantly whole form used in the ELISA. Accordingly, the validity of directly comparing data from ELISAs involving the two forms must be questioned.

35 mM Diethylamine (containing 35 mM NaCl) used for elution of antibody had a bleaching effect on the immobilised HXO. The suggested explanation for this is that the FAD moiety is stripped from HXO, given the effects of diethylamine on the enzyme seen in Chapter 3. Also, SDS-PAGE revealed that the immobilised HXO became highly fragmented following elution cycles. However, concerns about a concomitant loss in antigenic activity of the immobilised enzyme were allayed, as both the total protein yield and titre of the eluted antibody decreased only slightly with repeated use. It is feasible that, following the loss of integrity of the ligand, and a resultant loss of some antigenic determinants, "hidden" HXO epitopes may have become exposed. This would allow antibodies with a different array of paratopes to be purified, although the difficulty in predicting what conformation HXO adopts during the ELISA prevents our confirming this.
The persistence of bound immunoglobulin following elution with 4 M urea led to diethylamine (35 mM) becoming the preferred eluant. Initial studies (Section 3.3.3.) had identified urea at this concentration to be least disruptive to HXO integrity amongst eluants tested. However, the long periods required for elution of antibody produced the same bleaching of the immobilised HXO as did harsher eluants. When elution was attempted with 2.8 M MgCl₂ (data not shown) little protein (approximately 0.4 mg) was obtained, the persisting antibody was removed with 35 mM diethylamine. This latter eluant removed bound immunoglobulin, whilst permitting reuse of the column.

It has been shown that immobilised XO retains activity toward xanthine, albeit with a higher than normal Km (Coughlan and Johnson, 1973). Therefore, it is reasonable to assume that many of the epitopes of the free enzyme are retained in the immobilised form. One limitation of CNBr-activated Sepharose immobilisation, however, is that the covalent bonds formed between the ligand and matrix are relatively labile, therefore leading to leaking of the immobilised antigen. The efficacy of the column did decrease following multiple usage, although the overall amount of antibody purified (following recirculation of the antiserum) remained essentially unaffected.

Establishing the exact subcellular localisation of XO is central to elucidating the function of the enzyme. In endothelial and epithelial cell cultures XO was found to be located not only in the cytoplasm, but also on the external surface of the cell membrane. A perinuclear cytoplasmic location aligns with the postulate that XO is a source of ROS that activate nuclear transcription factors, e.g. NF-κB. Furthermore, the consistent punctate staining observed suggests a vesicular location, a finding confirmed by subsequent studies using a mouse monoclonal antibody to XO (Hoare, 2002). These vesicles possibly serve to store XO prior to its export from the cell. The asymmetry of the enzymes exterior localisation on these cells, possibly bound to glycosaminoglycans, is the first detailed evidence for such a localisation. Involvement in cell-cell signalling, incorporating the ability of XO to generate ROS, is a real possibility to explain this result. Therefore, the polyclonal antibody produced
in the current study proved to be a valuable and versatile tool for the routine detection of XO via Western Blots, ELISA and confocal microscopy.

The utility of antibodies in enzyme purification is open to debate. The purification technique is expensive insofar as it is time consuming and labour intensive. Nevertheless, the antibodies are relatively stable and, given recent improvements in purification of human milk XO, easily purified. The range of active/inactive forms present in different tissues complicates purification of XO by conventional means. However, the binding of polyclonal antibodies raised against XO is independent of activity, as they display avidity for the low activity human, and also the high activity bovine forms of the enzyme.
Chapter 5

Preparation and testing of a rabbit anti-(HXO) immunoaffinity column
5. PREPARATION AND TESTING OF A RABBIT ANTI-(HXO) IMMUNOAFFINITY COLUMN

5.1. INTRODUCTION

This chapter concerns the optimisation of the immunoaffinity technique to be used for purification of XO from tissue. A column technique, rather than batch chromatography, was employed because elution is usually more effective with this method. As the eluant passes through the column, any antigen-antibody interactions that "breathe" are more easily dissociated in a column system, as the progression of the elution front prevents any re-association (Harlow and Lane, 1988). Therefore, the elution conditions for the batch technique are generally harsher.

In order to establish its efficacy, the immunoaffinity column was tested with pure HXO, in addition to a crude form of the enzyme. Further to Section 3.3.3, the most appropriate eluant in terms of the recovery of active enzyme, and of column reusability, was also determined.
5.2. METHODS

5.2.1. CROSS-LINKING OF IgG TO PROTEIN A

Protein A was coupled to CNBr-activated Sepharose 4B as described previously (Section 4.2.1.1).

Rabbit anti-(HXO) IgG, prepared as in Section 4.2.2, was incubated with protein A gel at a concentration of 0.5 mg of antibody per ml of wet beads. The gel was allowed to mix for 1 h at room temperature with gentle rocking. Unbound antibody was removed by centrifugation (3,000 g, 5 min, at room temperature) in 10 volumes of 50 mM sodium borate, pH 9.0. This step was repeated before resuspending the beads in the same buffer. 20 mM Dimethylpimelimidate was added and the suspension rotated for 30 min at room temperature. The reaction was stopped by the addition of 0.2 M ethanolamine, pH 8.0, centrifuging (as above), and incubating in the same solution for 1 h at room temperature. The beads were washed in the same way with PBS, and stored in PBS containing 0.01% sodium azide.
5.2.2. Preparation of the Fc portion of Rabbit IgG

Rabbit IgG was cleaved to obtain the Fc fragments that were to be used to block non-reacted sites on the rabbit anti-(HXO) IgG-protein A matrix. The Fc fragments themselves have no means of binding antigen. Therefore, attachment of these fragments prevents the binding of IgG or any other protein with an affinity for protein A present in tissue homogenates. The following procedure is based on that of Harlow and Lane (1988).

Rabbit IgG was affinity-purified from normal rabbit serum (i.e. non-immunised, as described in Section 4.2.2.4) to a concentration of 5 mg/ml in 100 mM sodium acetate (pH 5.5). Cysteine and EDTA were added to final concentrations of 50 mM and 1 mM respectively. Papain (10 μg/mg antibody) was incubated with the solution for 8 h at 37°C. The reaction was stopped with the addition of 75 mM iodoacetamide and the solution was rotated for 30 min at room temperature. Separation of intact IgG from Fc/Fab was achieved by gel filtration on Sephadex G75. Prior to separation, the column was calibrated with blue dextran and DNP-lysine to determine the void and exclusion volumes, respectively.
5.3. RESULTS

5.3.1. TESTING OF THE RABBIT ANTI-(HXO) IMMUNOAFFINITY COLUMN WITH PURE HXO

Immunoaffinity gel containing a total amount of 12 mg rabbit anti-(HXO) polyclonal IgG was prepared as in Section 5.2.1. This matrix was divided into 3 equal portions and packed into columns (5 cm length x 1 cm diameter). 2 mg of purified HXO (Section 3) was diluted to 20 ml in PBS and circulated slowly on the column overnight to allow maximum binding. Unbound enzyme was washed off the column with PBS until the change in A_{280} was negligible. The attempted elution of pure HXO with either 4 M urea, 3 M KSCN or 35 mM diethylamine from separate columns is described below.

5.3.1.1. 4M UREA

The purified enzyme was diluted and circulated on the immunoffinity column as described above (Section 5.3.1). Approximately 1.5 mg of enzyme remained unbound following circulation, as determined by the dye-binding assay (Section 2.2.1). The 4 M urea-facilitated elution proved unsuccessful. HXO was not detected in the eluted fraction by protein assay and was therefore assumed to have remained bound to the immunoaffinity matrix.
5.3.1.2. 3 M KSCN

3 M KSCN was known to have a disruptive effect on the structure and activity of HXO (Section 3.2.5). Minimisation of the disruptive effects of KSCN during elution was attempted by connecting a PD-10 gel filtration column (Pharmacia; Sephadex G-25) in series with the immunoaffinity column (Figure 5.1), thereby immediately separating the eluant from the eluted enzyme. Both columns were equilibrated with PBS until the change in $A_{280}$ was negligible. Residual buffer was aspirated from the top of the immunoaffinity gel and replaced with 2 ml of 3 M KSCN. The apparatus was reconnected and elution was allowed to proceed with the KSCN being separated from the eluted enzyme immediately on exiting the immunoaffinity column (Figure 5.2).
Figure 5.1 Strategies used in immunoaffinity purification. (i) The standard method employed in this study and (ii) the strategy developed to minimise the effects of 3 M KSCN on purified HXO.
Figure 5.2 Elution profile from the rabbit anti-(HXO) immunoaffinity column with 3 M KSCN used as eluant. 1 = PBS wash of unbound HXO from the column. 2 = PBS equilibration of PD-10 gel filtration column in series. 3 = Application of 3 M KSCN. 4 = HXO elution (following gel filtration). 5 = 3 M KSCN exiting the PD-10 column following separation from eluted HXO.
A total of 0.4 mg HXO was eluted from the column (determined by protein assay; Section 2.2.1), which correlated with the amount of residual enzyme in the unbound fraction (1.54 mg). Structural and functional changes to the enzyme were analysed by 7.5 % SDS-PAGE (Fig 5.3), UV-visible absorption spectra (Fig 5.4) and by spectrophotometric assay (Table 5.1).

**Figure 5.3** A 7.5% SDS-PAGE of HXO following elution from a rabbit anti-(HXO) immunoaffinity column with 3 M KSCN. Lanes 1 and 6 contain molecular weight markers. Lanes 2 and 3 contain commercially available bovine XO. Lanes 4 and 5 contain HXO prior to and following immunoaffinity chromatography utilising 3 M KSCN as eluant, respectively.
**Figure 5.4** UV-visible absorption spectrum of pure HXO eluted with 3 M KSCN from an anti-(HXO) immunoaffinity column. "Pure HXO" is an absorption spectrum of HXO b) "3 M KSCN" is an absorption spectrum of pure HXO following elution from the immunoaffinity column with 3 M KSCN. Both spectra were recorded at the same protein concentration (approx 1.2 mg/ml).
No degradation or detectable alteration in the overall molecular weight of the eluted enzyme had occurred. However, the UV-visible spectral analysis was similar to that obtained in Section 3.2.5.3 for pure HXO exposed to 3 M KSCN. Following overnight dialysis, the spectrum still bore no resemblance to that typically associated with HXO. Following elution with 3 M KSCN 68% of the original activity toward xanthine was recovered.

### 5.3.1.3. **Effect of KSCN on the Reusability of the Immunoaffinity Column**

Further testing of the immunoaffinity column showed that elution with 3 M KSCN had damaged the column; immobilised antibody was no longer able to bind pure HXO. Therefore, this eluant was found to be unsuitable for the routine purification of XO from tissue and its use was discontinued.

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**Table 5.1** The activity of HXO toward xanthine following 3 M KSCN elution from rabbit anti-(HXO) column. Values are means ± SD (n=4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity (nmoles/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original HXO</td>
<td>86.5 (± 3.34)</td>
</tr>
<tr>
<td>3 M KSCN Eluted HXO</td>
<td>58.7 (± 2.85)</td>
</tr>
</tbody>
</table>
5.3.1.4. 35 mM DIETHYLAMINE

The elution strategy developed to minimise the effects of 3 M KSCN [Figure 5.1 (ii)] could not be used for this eluant due to the larger volume required to remove bound antigen. Loss of FAD from HXO on incubation with 35 mM diethylamine was thought to contribute to the observed loss in activity (Section 3.2.5). Therefore the effects of 35 mM diethylamine were minimised by the following method.

HXO eluted by 35 mM diethylamine (containing 35 mM NaCl) was collected in 0.8 ml fractions, each fraction tube contained 0.2 ml 1 mM FAD in 1 M Na-Bicine buffer (0.2 ml), pH 8.3 (containing 50 mM NaCl). Fractions containing protein were pooled (Figure 5.5) and dialysed against 50 mM Na-Bicine buffer, pH 8.3 (containing 50 mM NaCl).

As with the 3 M KSCN-facilitated elution (Section 5.3.1.2), 0.4 mg HXO was eluted from the column (determined by protein assay; Section 2.2.1). Elution produced no apparent change to the enzyme's molecular weight as determined by 7.5 % SDS-PAGE (Fig 5.6).
Figure 5.5 Elution profile from the rabbit anti-(HXO) immunoaffinity column, 35 mM diethylamine used as eluant. 1 = PBS wash of unbound HXO from the column. 2 = Application of 35 mM diethylamine. 3 = HXO elution. 4 = PBS wash following elution.
Figure 5.6. 7.5% SDS-PAGE of HXO following elution from a rabbit anti-(HXO) immunoaffinity column with 35 mM diethylamine (containing 35 mM NaCl). Lane 1 contains pure HXO subjected to immunoaffinity chromatography. Lane 2 contains pure HXO. Lane 3 contains molecular weight markers.
5.3.1.5. **Effect of FAD on Recovery of HXO Activity and Conformation**

One of the detrimental effects of exposure of HXO to 35 mM diethylamine is believed to be dissociation of the FAD group (Section 3.4). Previous studies demonstrated that most of the activity lost following flavin removal could be recovered on incubation of deflavoperoxidase with FAD (Komai et al., 1969; Kanda et al., 1972; Saito et al., 1989). Therefore, the following attempts were made to recover structural and functional integrity.

HXO eluted with 35 mM diethylamine was incubated for 2 h at 4°C in 1M Na-Bicine pH 8.3 (containing 50 mM NaCl) with or without 1 mM FAD. Excess FAD was removed by dialysis against several changes of 50 mM Na-Bicine buffer, pH 8.3 (containing 50 mM NaCl) and by PD-10 gel filtration (Pharmacia; Sephadex G-25) against the same buffer. The effects of FAD addition on activity and conformation of eluted HXO were determined by performing xanthine, pterin and NADH oxidase activity assays (Table 5.2) and UV-visible absorption spectra (Figure 5.7). Following overnight circulation, small changes to the UV-visible absorption spectrum of the enzyme (i.e. unbound HXO) were also observed (Figure 5.8).
### Table 5.2 Xanthine, pterin and NADH oxidase activity assays of HXO

Eluted enzyme activities, following incubation without ("Immunoaffinity Purified" HXO) and with 1 mM FAD ("Immunoaffinity Purified" HXO + 1 mM FAD), were compared to the original HXO. Values are means ± SD (n=3).

<table>
<thead>
<tr>
<th>Activity</th>
<th>Original HXO</th>
<th>&quot;Immunoaffinity Purified&quot; HXO</th>
<th>&quot;Immunoaffinity Purified&quot; HXO + 1 mM FAD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Activity (nmoles/min/mg)</td>
<td>Specific Activity (nmoles/min /mg)</td>
<td>% of Original</td>
</tr>
<tr>
<td>Xanthine</td>
<td>86.03 ± 1.21</td>
<td>29.09 ± 1.02</td>
<td>34</td>
</tr>
<tr>
<td>Pterin</td>
<td>23.30 ± 0.067</td>
<td>11.02 ± 1.17</td>
<td>47</td>
</tr>
<tr>
<td>NADH Oxidase</td>
<td>110 ± 2.19</td>
<td>37.16 ± 0.72</td>
<td>37</td>
</tr>
</tbody>
</table>
Figure 5.7 UV-visible absorption spectra of pure HXO following elution from an anti-(HXO) immunoaffinity column. HXO refers to pure human enzyme. Diethyl refers to enzyme eluted with 35 mM diethylamine (containing 35 mM NaCl) from the immunoaffinity column. FAD refers to eluted enzyme following overnight incubation with 1 mM FAD.
Figure 5.8 UV-visible absorption spectra of pure HXO following overnight circulation through an anti-(HXO) immunoaffinity column. "Original HXO" refers to an absorption spectrum of HXO b) "Circulated HXO" is an absorption spectrum of HXO following circulation overnight through the immunoaffinity column
5.3.1.6. **Effect of diethylamine on the reusability of the immunoadfinity column**

Re-testing of the immunoadfinity column was possible following elution with 35 mM diethylamine. There appeared to be no immediate significant loss in performance, as shown by successive elutions (Table 5.3).

<table>
<thead>
<tr>
<th>Amount of HXO circulated (mg)</th>
<th>Amount of HXO eluted (mg)</th>
<th>Unbound HXO (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td>0.412</td>
<td>1.50</td>
</tr>
<tr>
<td>2.50</td>
<td>0.401</td>
<td>2.02</td>
</tr>
<tr>
<td>0.80</td>
<td>0.405</td>
<td>0.37</td>
</tr>
</tbody>
</table>

*Table 5.3 Reusability of the immunoadfinity column following elution with 35 mM diethylamine. The amount of HXO in each case was determined by protein assay and reflects successive experiments on the same column (Section 2.2.1).*
5.3.1.7. **TESTING WITH CRUDE EXTRACT.**

In order to ensure the ability of the immunoaffinity column to remove HXO from a heterogeneous protein solution, the following experiment was performed.

A crude extract of HXO from human milk was prepared as in Section 3.2.1. The extract, following the second ammonium sulphate precipitation, was dialysed against PBS overnight. The dialysate was then circulated through the column and unbound protein washed off as described for the pure form of the enzyme (Section 5.3.1). Bound protein was eluted with 35 mM diethylamine (containing 35 mM NaCl) (Figure 5.9), as described in Section 5.3.1.4. Following dialysis, the purified enzyme was analysed by performing SDS-PAGE (Figure 5.10) and activity assays; the results of which were compared against enzyme purified by heparin chromatography (Section 3.2.2) from the same crude extract. The UV-visible absorption spectrum of the immunoaffinity purified enzyme was identical to that seen in Figure 5.7 for eluted enzyme following overnight incubation with 1mM FAD.
**Figure 5.9** Elution profile of HXO purified from a crude milk preparation, 35 mM diethylamine used as eluant. 1 = PBS wash of unbound HXO from the column. 2 = Application of 35 mM diethylamine. 3 = HXO elution. 4 = PBS wash following elution.
Figure 5.10 7.5% SDS-PAGE of HXO purified from crude milk extract following elution from a rabbit anti-(HXO) immunoaffinity column with 35 mM diethylamine (containing 35 mM NaCl). Lane 1 contains immunoaffinity purified HXO. Lane 2 contains heparin purified milk HXO. Lane 3 contains molecular weight markers.
5.3.2. **ATTEMPTED BLOCKING OF RABBIT ANTI-(HXO) IMMUNOAFFINITY MATRIX**

Non-specific binding (i.e. IgG) via unreacted sites on the protein-A may occur when the tissue homogenate is applied to the immunoaffinity column. In order to prevent this, an attempt was made to block those sites unoccupied with rabbit anti-(HXO) pAb by cross-linking Fc fragments from rabbit IgG.

5.3.2.1. **PREPARATION OF Fc FRAGMENTS OF RABBIT IgGS**

Fc fragments of rabbit IgG were prepared as in Section 5.2.2. Following gel filtration (Figure 5.11), the digested antibody was run on 10% SDS-PAGE to ensure purity (Figure 5.12). A single band at 25 kDa was obtained containing both Fc and Fab fragments. The Fc portions will bind to protein-A, so blocking excess reactive sites, whilst the Fab will have no affinity and remain in solution. The Fc fragments will then be cross-linked to the matrix as described in Section 5.2.1.
Figure 5.11 Gel filtration of papain-digested rabbit IgG. The peak at approximately 50 ml corresponds to Fc and Fab fragments. The peak at 25 ml is due to undigested IgG eluting in the void volume (approximately 32 ml). The rise in absorbance from 85 ml is due to DNP-Lysine eluting in the exclusion volume.
CHAPTER 5: PREPARATION AND TESTING OF A RABBIT ANTI-(HXO) IMMUNOAFFINITY COLUMN

5.3.2.2. TESTING OF IMMUNOAFFINITY COLUMN BLOCKED WITH Fc FRAGMENTS

An anti-(HXO) immunoaffinity gel was prepared as in Section 5.2.1. The Fc fragments were then cross-linked in the same way as the anti-(HXO) IgG (Section 5.2.1). HXO was circulated on the column overnight and unbound enzyme was washed off, as in Section 5.3.1. However, 35 mM diethylamine eluted no enzyme from the column. On testing the unbound fraction, HXO had been removed from the circulating solution, whilst the top of the immunoaffinity gel had brown colouring, which is indicative of bound HXO. Increasing the concentration of diethylamine (100 mM) and addition of DTT (5 mM) had no effect;
adsorbed enzyme remained bound. Therefore, the additional cross-linking of the Fc fragments appeared to have disrupted the immunoaffinity matrix so preventing elution of antigen.

5.3.2.3. **INTERACTION OF HXO WITH PROTEIN A**

In order to explore the possibility that HXO is removed from solution by protein A rather than by the anti-(HXO) IgG, the following experiment was performed.

Protein A (4 mg) was immobilised on CNBr-Sepharose 4B as described in Section 4.2.1.1. Purified HXO (2 mg) was diluted and circulated through the column as described previously (Section 5.3.1). Unbound protein was washed from the column until the change in $A_{280}$ was negligible and a protein assay (Table 5.4) then performed. 35 mM diethylamine (containing 35 mM NaCl) was passed through the column to elute any bound protein. Although a small increase in absorbance at 280 nm was detected, this was insignificant when compared to the amount eluted from the immunoaffinity columns as described previously (Section 5.3.1.4).

<table>
<thead>
<tr>
<th>HXO Sample</th>
<th>Amount of protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original HXO</td>
<td>0.5</td>
</tr>
<tr>
<td>HXO following circulation through protein-A</td>
<td>0.468</td>
</tr>
<tr>
<td>column</td>
<td></td>
</tr>
<tr>
<td>Eluted HXO</td>
<td>0.028</td>
</tr>
</tbody>
</table>

*Table 5.4 Protein assay on HXO prior to, and following, circulation on a protein-A affinity column.*
5.4. DISCUSSION.

This chapter describes the preparation and testing of an anti-(HXO) immunoaffinity column using rabbit anti-(HXO) polyclonal IgG antibodies.

The inactivating effects of 3 M KSCN on HXO were reduced by the addition of the gel filtration column in series with the immunoaffinity column. Original trials using 3 M KSCN reduced the xanthine oxidase activity of pure HXO to approximately 50% active (Section 3.3.3.5), this increased to 68% with the incorporation of the gel filtration step. However, spectral analysis still indicated a degree of denaturation, which may have been (partially) reversed on incubation with FAD. Nevertheless, the disruption to the column itself by this eluant proved irreparable and, consequently, its use was discontinued.

The use of 4 M urea can be discounted as it failed to remove any bound HXO. Although the column would have been re-usable, urea will N-terminally block protein. This is of consequence given that the protein purified by this method from human tissue was to be N-terminally sequenced in order to identify it as xanthine oxidase. Bound HXO in this experiment was subsequently eluted with 35 mM diethylamine (data not shown).

The eluant identified as the most suitable for purification of HXO from the immunoaffinity column was diethylamine. As reported previously (Section 3.4), an advantage this eluant has over others is that post-elution, the immunoaffinity column is able to be re-used - a prerequisite for the routine employment of a purification method.

Immediate neutralisation of eluted enzyme, along with incubation with flavin, reduced the detrimental effects of diethylamine during elution. A rise in recovered xanthine oxidase (34 to 50% of original), pterin oxidase (47 to 53%) and NADH oxidase (37 to 74%), compared to those obtained without incubation with FAD (Table 5.2), were consistent. Although differences persist in the UV-visible absorption spectra between immunoaffinity and heparin
purified HXO, some of this may be attributed to the overnight recirculation of the enzyme, as seen in Figure 5.8.

Purification of HXO from crude milk extract was effected. The results highlight the advantages of using heparin as a ligand for HXO purification from milk when compared to the immunoaffinity method used here. However, tissue homogenates will contain far more proteins that have affinities for glycosaminoglycans, although use may be made of heparin chromatography as an intermediate stage during purification.

The attempted blocking of unreacted protein-A sites on the immunoaffinity matrix by Fc fragments proved unsuccessful. Without such blocking, any IgG present in tissue homogenate will co-purify with the antigen to the immobilised antibody. It appears that the extra stage involving linkage of Fc fragments in some way modifies the immobilised antibody. The pure enzyme used to test the matrix was removed from solution by this modified column; however, elution of this enzyme could not be achieved even by inclusion of DTT in the elution buffer. DTT was included to minimise aggregation of HMXO which is susceptible to aggregation when present in the O form (Godber, 1998). DTT facilitates the O to D conversion thereby disaggregating the enzyme. It is worth pointing out that, although DTT was included in an effort to remove the bound HXO, this should be avoided at higher concentrations. The result of its use would be reduction of the disulphide bridges between heavy and light chain of the immobilised IgG, thereby inactivating the column. In previous immunoprecipitation experiments, attempts to block non-specific sites with Fc fragments have yielded similar disappointing results (personal communication, Dr. Mustapha Benboubetra). Such blocking was not further explored.

It was established that protein A would not remove HXO from solution, although it may have affinity for other non-immunoglobulin protein in tissue homogenate.
Chapter 6

Attempted purification of xanthine oxidase from human tissue
6. **ATTEMPTED PURIFICATION OF XANTHINE OXIDASE FROM HUMAN TISSUE**

6.1. **INTRODUCTION**

This chapter details the attempts made to purify XO from human liver and heart tissue. There is considerable interspecies variation of XO activity in heart tissue (Section 1.1.8), which prevents the extrapolation of findings in animals to the human situation.

It was not until 1986 that evidence regarding the activity in human tissue became available (Krenitsky et al., 1986). As described in Section 1.1.7; XO activity in human liver was reported to be similar to that found in bovine milk. Liver has been most studied regarding activity and presence of the enzyme, which in bovine tissues appears to be confined to the capillary endothelium (Jarasch et al., 1981), although this localisation has not been confirmed in humans. A study by Linder et al. (1999) suggested that liver XO was situated in periportal hepatocytes and Kupfer cells. No XO protein was detected in the latter cell type in an earlier study (Moriwaki et al., 1993).

Xu et al., (1994) demonstrated the expression of human XDH gene in all tissues studied by using Northern blot analysis. In 1998, Saksela et al. quantified XO mRNA expression and activity in developing human tissues and saw the strongest signal in the intestine and liver. Although several orders of magnitude lower, XO mRNA was detected in human heart.
Reported activities of XO in human heart range from high (Wajner and Harkness, 1989), to low (de Jong et al., 1990; Muxfeldt and Schaper, 1987; Abadeh et al., 1993) to undetectable (Eddy et al., 1987; Grum et al., 1989; Podzuweit et al., 1991). Hellsten-Westing (1993) observed immunoreactivity of HXO in vascular smooth muscle cells and capillary endothelial cells as well as in the smaller vessels of the cardiac muscle, but not in endothelium lining larger vessels. It is conceivable that the occurrence of XO in the capillary endothelium is a consequence of distress-related release from the liver of enzyme, which then binds to the microvasculature via sulphated glycosaminoglycans (Adachi et al., 1993).

The recent failure to detect XO in normal (i.e. non-diseased) human heart using rabbit anti-HXO pAbs adds to the confusion regarding its occurrence in this tissue (Linder et al., 1999). However, successful detection of XO in human heart by the use of immunoaffinity techniques (Abadeh et al., 1992; Abadeh et al., 1993; Hellsten-Westing, 1993; Moriwaki et al., 1993) suggests this to be the recommended method for purification of HXO from human tissue. This chapter outlines this approach together with the use of heparin chromatography in the purification of XO from human tissues.
CHAPTER 6: ATTEMPTED PURIFICATION OF XANTHINE OXIDASE FROM HUMAN TISSUE

6.2. METHODS

6.2.1. HOMOGENISATION OF HUMAN TISSUE

The procedure for the homogenisation of human tissue was based upon that used by Hellsten-Westling (1993).

When handling human tissue, the necessary safety precautions were adhered to. A Class II safety cabinet was utilised for this work.

Whole deep frozen tissue (stored at -70°C for up to 3 months) was weighed and placed in liquid nitrogen for 5 min before being shattered. Approximately 10 g of frozen human liver or 60 g (10%) of frozen human heart was used to prepare a crude homogenate. Following thawing, the tissue was chopped into small pieces prior to homogenisation in 5 vols of ice cold PBS, containing 5 mM EDTA, 5 mM DTT and protease inhibitors (leupeptin, antipain, aprotinin and pepstatin A (all at 1 µl/ml). The crude homogenate was centrifuged at 26,700 g for 45 min at 4°C. The pellet was then discarded and the supernatant (known subsequently as the "initial crude homogenate") was stirred for 45 min with 20% (w/v) ammonium sulphate. The mixture was then centrifuged (15,000 g, 30 min, 4°C), the solid precipitate discarded and the supernatant stirred with an additional 20% (w/v) ammonium sulphate as above. The crude XO preparation was obtained as a precipitate following centrifugation (15,000 g, 30 min, 4°C). This pellet was resuspended in PBS containing EDTA, DTT and protease inhibitors as described for the homogenisation solution. Dialysis against several changes of this PBS was performed over the following 24 hours. Alternatively, for Heparin chromatography the homogenate was dialysed against several changes of "heparin buffer" (25 mM sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and protease inhibitors as described for the homogenisation solution). The dialysate
was centrifuged at 26,700 g (1 h, 4°C) before filtering through a 0.22 μm disposable filter. This preparation was used immediately.

6.2.2. ELISA FOR THE DETECTION AND DETERMINATION OF HXO

ELISA kits for the quantitative determination of HXO were obtained from Dr Eugene Bosmans, Eurogenetics, Belgium. All reagents and coated microtitre strips were supplied with the kits and used as follows:

"Zero Standard/Sample Diluant" (1 ml) was used to reconstitute 80 ng lyophilised HXO. This solution was then diluted with the same buffer to form a standard curve of HXO (40, 20, 10, 5, 2.5, 1.25 ng/ml). These standards were pipetted in duplicate, to separate wells of microtitre strips coated with rabbit anti-(HXO) polyclonal antibodies (120 μl/well). Samples to be assayed were diluted as required in the same buffer and added separately in triplicate (120 μl/well). The microtitre-plate was sealed before incubating for 2 h at 37°C, following which it was washed 5 times with "Washing Buffer" using a Tecan plate washer. A 100 μl aliquot of biotin-rabbit anti-(HXO) polyclonal conjugate was added to each well, the plate resealed and incubated for 1 h at 37°C. The washing cycle was repeated before adding Streptavidin-HRP conjugate (100 μl/well). The microtitre plate was then resealed and incubated for 20 min at 37°C. "Solution A" (citrate phosphate buffer containing peroxide at working strength) was mixed with an equal volume of "Solution B" (TMB at working strength) and, following a final washing cycle, 200 μl was added to each well. The plate was again resealed and incubated for 20 min at room temperature in the dark. The reaction was attenuated with the addition of 50 μl "Stopping Solution" (2N H₂SO₄) and the absorbance of each well was determined at 450 nm using a Tecan Spectra microtitre-plate reader.

Figure 6.1 is a typical standard curve obtained for purified milk HXO.
Figure 6.1 ELISA standard curve obtained with purified human milk HXO. Values are ± SD (n=3).
6.2.3. **WESTERN BLOTTING – IMMUNOPROBING FOR HXO**

Western blotting was performed as described earlier (Section 2.2.4) with the following modification:

Following electrotransfer and blocking, the membrane was immunoprobed with an anti-(HXO) monoclonal antibody (kindly donated by Dr Eugene Bosmans, Eurogenetics) specific for the human enzyme, i.e. no cross-reactivity with the bovine XO was observed (discussion with Dr Mustapha Benboubetra). The above antibody was diluted 1:1000 times in TBST containing 1% BSA, applied to the blot and incubated at 37°C for 1½ h. The membrane was then washed with TBST (as described in Section 2.2.4.2). The secondary antibody (anti-mouse-IgG peroxidase conjugate) was diluted 1:500 times and applied to the blot (1 h, room temperature).

The membrane was washed and visualisation of the bands performed as in Section 2.2.4.2.
6.3. RESULTS

6.3.1. HEPARIN CHROMATOGRAPHY

Extension of the successful human milk XO purification by heparin chromatography to the purification of the enzyme from human tissue is an obvious approach. The conditions used for heparin chromatography with human tissue were essentially the same as those for human milk XO purification (Section 3.2.2), with the addition of proteinase inhibitors in each elution buffer (leupeptin, antipain, aprotinin and pepstatin A) all at 1 μl/ml.

A 10 ml column of heparin bound to 4% crosslinked agarose was washed before use with heparin buffer. The crude homogenate was applied slowly to the column and impurities were removed with heparin buffer, followed by the same buffer containing 0.08 M NaCl, until the A_{280} of the eluant reached a constant baseline. Heparin buffer containing 0.4 M NaCl was then applied to the column and eluted fractions were pooled and dialysed against heparin buffer containing proteinase inhibitors.
6.3.1.1. **HUMAN LIVER**

Homogenates of post mortem human liver were prepared as in Section 6.2.1. The activity toward pterin was assayed throughout purification (Table 6.1). Remarkably, the total activity toward pterin increased during the ammonium sulphate precipitation stages of purification, whilst the total amount of protein decreased in both steps. The heparin stage proved successful insofar as 98% of the activity applied to the column was recovered on elution with 0.4 M NaCl (Figure 6.2). The remaining activity appeared in the unadsorbed and 0.08 M NaCl fractions (<1% of the total activity applied, respectively).

ELISA analysis (Section 6.2.2) revealed that 60 μg (CV = 1.68%; n = 3) of XO protein was partially purified from 10 g of human liver tissue. Consequently, the specific activity of the partially purified human liver XO was determined to be 677.3 nmoles pterin/min/mg. Although the amount of HXO protein in the initial crude homogenate was determined to be very similar (63 μg), it seems implausible that >95% of the original protein could be purified from a crude homogenate. However, the aforementioned apparent increase in activity during the purification appears to be reflected in the ELISA data.

SDS-PAGE analysis highlights the impurity of the eluted enzyme (Figure 6.3.a) although XO protein could indeed be detected by Western blot (Figure 6.3.b; see Section 6.2.3). In both the crude liver homogenate and the 0.4 M NaCl eluted fraction, a faint band at 155 kDa (corresponding to the monomeric protein) and a major band at approximately 140 kDa were identified.
Figure 6.2 Profile of human liver homogenate eluting from a heparin affinity column. The peak at 0.4 M NaCl corresponds to the elution of 98% of the pterin activity applied to the column.
CHAPTER 6: ATTEMPTED PURIFICATION OF XANTHINE OXIDASE FROM HUMAN TISSUE

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity (nmol/min)</th>
<th>% CV (n = 3)</th>
<th>Yield (%)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Crude Homogenate</td>
<td>3.84</td>
<td>3.1</td>
<td>100</td>
<td>469</td>
<td>0.0082</td>
<td>1</td>
</tr>
<tr>
<td>Following 1st Amm. Sulph</td>
<td>5.6</td>
<td>6.3</td>
<td>146</td>
<td>294</td>
<td>0.019</td>
<td>2.32</td>
</tr>
<tr>
<td>Following 2nd Amm. Sulph</td>
<td>41.5</td>
<td>4.6</td>
<td>1081</td>
<td>166</td>
<td>0.249</td>
<td>30.4</td>
</tr>
<tr>
<td>0.4 M NaCl Elution</td>
<td>40.8</td>
<td>5.7</td>
<td>1062</td>
<td>29.1</td>
<td>1.4</td>
<td>171</td>
</tr>
</tbody>
</table>

Table 6.1 Purification of Human liver XO by heparin chromatography. A 10 g sample of human liver was used for this study. The total amount of protein at each stage was determined by the Bradford method (Section 2.2.1). The activity was determined by pterin assay (Section 2.2.2.3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>HXO Protein Conc. (µg/ml)</th>
<th>% CV (n=3)</th>
<th>Vol (ml)</th>
<th>Total HXO Protein (µg)</th>
<th>Total Activity (nmol pterin/min)</th>
<th>Specific Activity (nmol pterin/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Crude Homogenate</td>
<td>1.226</td>
<td>16.1</td>
<td>51.5</td>
<td>63.14</td>
<td>3.84</td>
<td>60.82</td>
</tr>
<tr>
<td>0.4 M NaCl Elution</td>
<td>0.496</td>
<td>1.68</td>
<td>121.5</td>
<td>60.23</td>
<td>40.8</td>
<td>677.3</td>
</tr>
</tbody>
</table>

Table 6.2 ELISA determined HXO protein content in human liver preparation following heparin chromatography. ELISA was performed on the preparation detailed in Table 6.1.
Figure 6.3  a) 7.5% SDS-PAGE and b) Western Blot of human liver homogenate subjected to heparin chromatography. Lane 1 contains molecular weight markers; Lane 2 contains purified milk HXO; Lane 3 contains human liver homogenate; Lane 4 contains fractions eluted with 0.4 M NaCl.
6.3.1.2. **HUMAN HEART**

Homogenates of post mortem cardiomyopathic human heart were prepared as in Section 6.2.1. Unlike human liver, pterin activity of the heart homogenate could not be detected. Assuming the same conditions as for human liver, XO elution was attempted with 0.4 M NaCl (Figure 6.4), although no activity was subsequently detected by pterin assay. Eluted HXO protein was not identified by Western Blot (Figure 6.5.b; see Section 6.2.3). ELISA for HXO was not performed.
Figure 6.4 Profile of human heart homogenate eluting from a heparin affinity column.
Figure 6.5  a) 7.5% SDS-PAGE and b) Western Blot of human heart homogenate subjected to heparin chromatography. Lane 1 contains molecular weight markers. Lane 2 contains purified milk HXO. Lane 3 contains human heart homogenate. Lane 4 contains fractions eluted with 0.4 M NaCl.
6.3.2. IMMUNOAFFINITY CHROMATOGRAPHY

Immunoaffinity gel containing a total of 8 mg of rabbit anti-(HXO) polyclonal IgG was prepared as in Section 5.2.1. This was divided into two equal portions and packed into two separate columns (5 cm length x 1 cm diameter). These were equilibrated with 10 volumes of PBS. The columns were used exclusively for one tissue type (i.e. liver or heart) to prevent cross contamination.

Homogenates of human tissue (deep frozen at -70°C for up to 3 months) were prepared as in Section 6.2.1 and were applied to the column; this was slowly circulated overnight. Non-specific protein was removed from the gel using PBS containing 5 mM EDTA, 5 mM DTT and protease inhibitors (leupeptin, antipain, aprotinin and pepstatin A (all at 1 μl/ml) followed by this PBS containing 1.0 M NaCl. Bound protein was eluted with 35 mM diethylamine containing 35 mM NaCl. Fractions of 0.8 ml were collected in tubes that contained 0.2 ml Na-Bicine buffer (1 M, pH 8.3; 35 mM NaCl) and FAD (1 mM). Following incubation for 1 h in this buffer, eluted protein fractions were pooled and dialysed against several changes of Na-Bicine buffer (0.05 M, pH 8.3; 20 mM NaCl). This procedure was repeated until the change in $A_{280}$ on elution was negligible, indicating that no further protein in the crude homogenate could be absorbed.
6.3.2.1. **Human Liver**

Homogenates of post mortem human liver were prepared as in Section 6.2.1 and immunoaffinity chromatography of this was performed as in Section 6.3.2.

The activity toward pterin was assayed throughout purification (Table 6.3). As with the liver preparation subjected to heparin chromatography (Section 6.3.1.1), the total activity increased during the ammonium sulphate precipitation stages of purification, whilst the total amount of protein decreased. However, although the final yield of HXO (as determined by ELISA; Table 6.4) was similar to that purified by heparin chromatography (Table 6.2), the specific activity of the HXO was lower.

An SDS-PAGE of the eluted protein is shown in Figure 6.6.a. Curiously, unlike that seen with the heparin chromatography technique, no bands corresponding to the monomeric human enzyme bands in either the crude homogenate or the adsorbed fraction were seen. However, major bands at 140, 85, 65 and 50 kDa were displayed. Subsequent immunoprobing (Figure 6.6.b) indicated that only the band at 140 kDa correlated to HXO.
CHAPTER 6: ATTEMPTED PURIFICATION OF XANTHINE OXIDASE FROM HUMAN TISSUE

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity (nmol/min)</th>
<th>% CV (n = 3)</th>
<th>Yield</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial crude</td>
<td>3.42</td>
<td>3.7</td>
<td>100</td>
<td>456</td>
<td>0.0075</td>
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<td>Following 1st Amm. Sulph.</td>
<td>6.38</td>
<td>3.7</td>
<td>187</td>
<td>222</td>
<td>0.028</td>
<td>3.83</td>
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<tr>
<td>Following 2nd Amm. Sulph</td>
<td>35.1</td>
<td>4.1</td>
<td>1026</td>
<td>149</td>
<td>0.236</td>
<td>31.4</td>
</tr>
<tr>
<td>35 mM Diethylamine</td>
<td>16.6</td>
<td>11.2</td>
<td>485</td>
<td>3.98</td>
<td>4.17</td>
<td>556</td>
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<tr>
<td>Elution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3 Partial purification of human liver XO by immunoaffinity chromatography. 10 g of human liver was used for this study. The total amount of protein at each stage was determined by the Bradford method (Section 2.2.1). The activity was determined by pterin assay (Section 2.2.2.3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>HXO Protein Conc. (µg/ml)</th>
<th>% CV (n=3)</th>
<th>Vol (ml)</th>
<th>Total HXO Protein (µg)</th>
<th>Total Activity (nmol pterin/min)</th>
<th>Specific Activity (nmol pterin/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Crude</td>
<td>0.944</td>
<td>13.88</td>
<td>63.2</td>
<td>59.69</td>
<td>3.72</td>
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<td>Homogenate</td>
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</tr>
<tr>
<td>35 mM Diethylamine</td>
<td>5.43</td>
<td>3.10</td>
<td>10</td>
<td>54.30</td>
<td>16.6</td>
<td>305.7</td>
</tr>
<tr>
<td>Elution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.4 ELISA determined HXO protein content in human liver preparation following immunoaffinity chromatography. ELISA was performed on the preparation detailed in Table 6.3.
Figure 6.6 a) 7.5% SDS-PAGE and b) Western blot of human liver homogenate subjected to immunoaffinity chromatography. Lane 1 contains molecular weight markers. Lane 2 contains crude human liver homogenate. Lane 3 contains adsorbed fractions eluted with 35 mM diethylamine (containing 35 mM NaCl). Lane 4 contains purified milk HXO.
6.3.2.2. **HUMAN HEART**

Homogenates of post mortem cardiomyopathic human heart were prepared as in Section 6.2.1 and immunoaffinity chromatography was performed as in Section 6.3.2. Following overnight dialysis, the eluted protein was concentrated prior to analysis.

Attempts were made to follow the activity towards pterin throughout purification. As with the heparin purification (Section 6.3.1.2), no activity was found in any of the stages prior to immunoaffinity purification. However, unlike the heparin-mediated purification, activity was demonstrated following the chromatography stage (Table 6.5), albeit considerably lower than that found in the liver tissue (Section 6.3.2.1). No activity was detected in the unadsorbed fraction.

The amount of HXO protein in the adsorbed fraction was determined by ELISA to be approximately 0.564 μg/g of whole heart tissue (Section 6.2.2). The average recovery of HXO protein from the crude heart homogenate by immunoaffinity chromatography was 77.5% (CV = 8%). The average specific activity of the eluted adsorbed fraction was 0.380 nmoles isoxanthopterin/min/mg (CV = 25%). Based on the amount of HXO protein detected by ELISA in the crude homogenate, the total amount of HXO protein present is approximately 0.7 μg/g of whole heart tissue, corresponding to a total of 445 μg in the heart used for this study.

The eluted protein was subjected to SDS-PAGE (Figure 6.7.a). No bands corresponding to that of the monomeric human enzyme were apparent. However, major bands at 140, 85, 60 and 50 kDa were displayed. Figure 6.7. b shows the eluted band at 140 kDa and the monomeric protein band of purified human milk XO enlarged.
A blot of a duplicate gel was probed as described in Section 6.2.3. This antibody recognised only the band at 140 kDa of the total eluted protein, as well as those of purified human milk XO at 155 and 140 kDa (Figure 6.8.). No protein bands of the crude heart homogenate were recognised.

Further gels (e.g. Figure 6.9) demonstrated that there were four main proteins present. A monomeric band at 155 kDa and further bands at 90, 50 and 25 kDa were obtained. Western analysis revealed the band at 50 kDa to be IgG (Figure 6.10). This immunoglobulin may have been co-purifying human IgG, binding to unoccupied sites on the protein A gel, or rabbit IgG leaching from the column during purification. The origin, however, was not established. Use of rabbit anti-(HXO) pAb indicated the band at 90 kDa to be a fragment of HXO. The band at 25 kDa also appeared to be a fragment of HXO (Figure 6.10, A), although this was not reflected when the blot was probed for IgG as well as HXO (Figure 6.10 B).
## Table 6.5 Purification of XO from human heart tissue.
The immunoaffinity chromatography stage resulted in 78% (CV = 8%; n = 3) of the HXO protein contained in the crude homogenate being recovered. The above was based on three separate preparations of the same heart; the total wet weight of the human heart tissue was 635 g.

<table>
<thead>
<tr>
<th>Preparation Number</th>
<th>Sample</th>
<th>Total Protein (wet weight)</th>
<th>HXO Content (µg)</th>
<th>Protein Yield (%)</th>
<th>Specific Activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preparation 1</strong></td>
<td>Tissue</td>
<td>60.52 g</td>
<td>Undetectable</td>
<td>N/A</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>Crude Homogenate</td>
<td>2490 mg</td>
<td>46.03 (CV = 6.1%) (n = 3)</td>
<td>100</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>Eluted Adsorbed Fraction</td>
<td>11.42 mg</td>
<td>32.91 (CV = 7.7%) (n = 3)</td>
<td>71</td>
<td>0.388 (CV = 21%) (n = 3)</td>
</tr>
<tr>
<td><strong>Preparation 2</strong></td>
<td>Tissue</td>
<td>62.34 g</td>
<td>Undetectable</td>
<td>N/A</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>Crude Homogenate</td>
<td>1843 mg</td>
<td>41.03 (CV = 13.2%) (n = 3)</td>
<td>100</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>Eluted Adsorbed Fraction</td>
<td>12.22 mg</td>
<td>38.45 (CV = 7.6%) (n = 3)</td>
<td>84.1</td>
<td>0.279 (CV = 1.1%) (n = 3)</td>
</tr>
<tr>
<td><strong>Preparation 3</strong></td>
<td>Tissue</td>
<td>65.21 g</td>
<td>Undetectable</td>
<td>N/A</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>Crude Homogenate</td>
<td>2076 mg</td>
<td>45.52 (CV = 11.6%) (n = 3)</td>
<td>100</td>
<td>Undetectable</td>
</tr>
<tr>
<td></td>
<td>Eluted Adsorbed Fraction</td>
<td>11.07 mg</td>
<td>35.00 (CV = 14.3%) (n = 3)</td>
<td>76.9</td>
<td>0.472 (CV = 10%) (n = 3)</td>
</tr>
</tbody>
</table>
Figure 6.7 a) 7.5% SDS-PAGE of human heart homogenate subjected to immunoaffinity chromatography. Lane 1 contains molecular weight markers. Lane 2 contains crude human heart homogenate. Lane 3 contains adsorbed fractions eluted with 35 mM diethylamine (containing 35 mM NaCl). Lane 4 contains purified milk HXO.

b) Highlighted area enlarged to show bands approximating to HXO.
Figure 6.8  a) Western blot of human heart homogenate subjected to immunoaffinity chromatography. Lane 1 contains molecular weight markers. Lane 2 contains crude human heart homogenate. Lane 3 contains adsorbed fractions eluted with 35 mM diethylamine (containing 35 mM NaCl). Lane 4 contains purified milk HXO.

b) Highlighted area enlarged to show bands approximating to HXO.
Figure 6.9 10% SDS-PAGE of immunoaffinity purified human heart preparation. Lanes 1 and 8 contain molecular weight markers. Lane 2 contains purified human milk XO (1μG). Lanes 3-7 contain rabbit anti-(HXO) pAb adsorbed fractions (2, 4, 6, 8, & 10 μg, respectively).
Figure 6.10 A Western blot of immunoaffinity purified human heart preparation probing for HXO and IgG.

A – Immunoprobing for HXO (using immunoaffinity purified rabbit anti-(HXO) pAb).

B – Immunoprobing for HXO and IgG (using immunoaffinity purified rabbit anti-(HXO) pAb and anti-human IgG antibodies).

Lanes 1 and 9 contain molecular weight markers.

Lanes 2 and 8 contain human IgG.

Lanes 3 & 7 and 4 & 8 contain rabbit anti-(HXO) pAb adsorbed fractions of human heart preparations (two separate heart preparations).

Lane 5 contains purified human milk XO (1µg). This had been incubated at room temperature for 12 hr prior to SDS-PAGE.
6.4. DISCUSSION

This chapter describes the use of the anti-(HXO) immunoaffinity column for the attempted purification of HXO from human tissue, especially the heart. However, heparin chromatography, which has proved successful when purifying human milk XO, was used initially.

6.4.1. HEPARIN CHROMATOGRAPHY

Although XO activity was recovered from the human liver homogenate, the eluted fraction was by no means pure. When used in tandem with the ELISA, this method did provide a means of determining the recovered activity, assuming that the enzyme is unaffected during purification. Curiously, the amount of activity present appeared to increase during the initial stages of purification. Previous studies have reported a co-purifying high molecular weight inhibitor of XO in bovine milk and rabbit heart (Bray, 1975 and Terada et al., 1994, respectively). Hellsten-Westing (1993) also reported a co-purifying 200 kDa protein that peptide mapping showed to be unrelated, although possibly associated to XO. It is a possibility that this protein may have been removed early in the preparation, thereby releasing previously inhibited activity. The presence of an inhibitor may also be reflected in the ELISA. The apparently high proportion of purified enzyme compared to that present in the initial crude extract may be artefactual in that an inhibitor may have masked epitopes that are later uncovered. Such an inhibitor is unlikely to be that described by Lee (1973), which precipitated at 60% saturation and is unlikely therefore, to have been removed from the preparation at the first ammonium sulphate step as in my purification procedure.
Endogenous hypothanxine may also be present in crude homogenate, inhibiting pterin activity. Incorporation of a Sephadex G-25 chromatography step is recommended to remove this prior to performing the pterin assay (Beckman et al. 1989) and some researchers have reported increased activity in rat heart homogenates following this stage (Schoutsen et al., 1983; Podzuweit et al., 1991). However, Eddy and co-workers (1987) found no evidence for the presence of endogenous inhibitors in human heart homogenates, whilst other groups reported the inclusion of this step made no difference when assaying liver homogenates from rat (Parks et al., 1988) and human (Sarnesto et al., 1996). In the present study, samples used for assaying from each stage of the purification were dialysed overnight, which should have been sufficient to dilute any endogenous purine below its $k$, removing the need to incorporate the Sephadex G-25 chromatography step.

Haemoglobin is known to quench the fluorescent signal produced by isoxanthopterin. The removal of haemoglobin during purification may also explain the increase in total activity observed. However, this effect should be circumvented by the addition of isoxanthopterin as an internal standard after inhibition of the reaction with allopurinol (Beckman et al. 1989).

The partially purified human liver enzyme was of very high activity when compared to the human milk enzyme purified earlier in this study (Section 3.3.1). Although elution occurred at the same ionic strength, the liver enzyme had a specific activity 25 times that of the milk enzyme. An ELISA of liver homogenate indicated the XO protein concentration to be 131 ng/mg (CV = 1.9 %) of total liver protein, which falls within the range (146 ± 70 ng/ml; CV = 49 %) reported by Sarnesto et al. (1996), an amount also determined by ELISA using a polyclonal antibody raised against milk HXO.
A potential drawback of using heparin chromatography for the purification of XO from tissue is that certain proteinases adhere to sulphated glycosaminoglycans, whereby they might be concentrated in close vicinity to XO. Although the adsorbed fraction was by no means pure, on Western blotting, the anti-(HXO) mAb did detect bands at 155 and 140 kDa. These findings compare favourably to those of Krenitsky et al. (1986) and), whose purified human liver XO yielded SDS-PAGE protein bands at 150, 135, 95, 55 & 38 kDa, while in 1993 Hellsten-Westing used a specific monoclonal antibody to precipitate XO protein from human liver to yield bands of 155, 143 and 95 kDa. Furthermore, Samesto et al. (1996) also detected 143, 125 & 87 kDa XO protein in liver homogenates. This suggests that, in the present study, proteinase activity was sufficiently inhibited to have had a minimal effect on enzyme degradation.

When crude heart homogenate was subjected to heparin chromatography, pterin assay and Western blotting failed to detect XO activity and protein, respectively, in either the crude homogenate or in the 0.4 M NaCl eluted fraction.

A complication of using heparin results from non-specific binding of protein in the crude homogenate. The column therefore becomes overloaded very quickly, greatly extending the time for purification.

6.4.2. IMMUNOAFFINITY CHROMATOGRAPHY

Immunoaffinity chromatography of crude human liver homogenate yielded approximately the same amount of enzyme as the heparin chromatography stage, although with increased purity. As expected, the activity of the partially purified enzyme was lower than that obtained by heparin chromatography due to the exposure to 35 mM diethylamine. The immunoaffinity-purified sample had 45% of the specific activity
toward pterin compared to the heparin-purified sample. This is comparable to the 53% recovered activity seen with diethylamine treated milk enzyme (Section 5.3.1.3.1).

An anomaly was the failure to detect, by Western blot, a monomeric band in the crude initial homogenate and the adsorbed liver fraction following immunoaffinity chromatography. The reason for this inability to detect any band in the crude homogenate is unclear, as the initial crude homogenate used for heparin chromatography had previously indicated the presence of the HXO protein. Possible degradation due to lack of protease inhibitors may be one explanation. Another possibility is degradation by the inhibitor-resistant protease reported by Saksela (1999). Present in the mitochondrial intermembrane space of human liver subcellular fractions, this novel type of proteolytic enzyme could have been released during liver homogenisation. The extra time required to perform immunoaffinity purification might have given these proteases further opportunity to degrade XO in the crude homogenate. Degradation in the adsorbed fraction may also be a consequence of the overnight circulation of the homogenate, whereby the liver enzyme is further exposed to extracellular and intracellular proteinases. In the case of milk, the enzyme is associated with the milk fat globule and so possibly less vulnerable.

The most important result is that active enzyme was purified from human heart. The enzyme demonstrated extremely low activity toward pterin. Heart HXO was 70 times less active than milk enzyme, even bearing in mind that diethylamine will have reduced this activity by approximately 50%. The failure of previous groups to detect XO activity in the human myocardium may therefore be explained by this low activity. The activities reported for human heart enzyme by previous groups ranges from 0.3-37 mU/g wet weight of tissue (Muxfeldt and Schaper, 1987; Wajner and Harkness, 1989; de Jong et al., 1990), where 1 unit (U) corresponded to the formation of 1 µmol urate/minute. In the present study, the activity was found to be 0.28 µU/g wet weight
(where 1 unit (U) corresponded to the formation of 1 μmol isoxanthopterin/minute). The pterin activity of human milk XO is approximately \(\frac{1}{4}\) that of its xanthine activity. Even accounting for this ratio for heart enzyme, there is still a considerable discrepancy between the previously reported values and that found currently. Price (1997) postulated that deep freezing may have an adverse effect on the activity of the heart enzyme in potential explanation of the fact that many groups previously failed to detect its presence. What also becomes apparent is that the enzyme present must surely be endogenous to the heart. If it were present solely following release from other tissue followed by its subsequent binding to sulphated glycosaminoglycans, the detected activity would be higher. Recently the specific activity of circulating XO towards pterin was reported to be approximately 100 nmol/min/mg (Harrison, 2002), considerably greater than that determined for heart XO in the current study. This variation in activity between milk, liver, heart and serum gives greater credence to the belief that XO has a tissue specific role.

The immunoglobulin detected in the heart preparation is probably endogenous, multi specific IgG, co-purifying as a result of the failure to block unoccupied protein A sites in the matrix. Another possibility is that it may be rabbit anti-(HXO) released from the column matrix as a result of the action of proteinases present, or through natural column degradation. However this latter theory may be rejected following the experiment described previously (Section 5.3.1.3.2). A further intriguing possibility is that the antibody may be endogenous human anti-(HXO) auto-antibody. Evidence for this has been provided by Clare and Lecce (1991) who reported a co-purifying immunoglobulin bovine milk XO preparations. The authors postulated that this antibody may explain the discrepancies between reported immunohistological association of XO in heart capillary endothelial cells and the absence of detectable enzymatic activity. As the hearts used in this study were known to be diseased, this possibility cannot be ruled
out. However, a previous report suggested that in this case IgM class antibodies would predominate (Harrison et al., 1990).

The heparin stage cannot be used to remove IgG from the adsorbed fraction following elution as reports suggest that this class of antibody has an affinity for heparin (Newman et al., 1998). Furthermore, spontaneously occurring auto-antibodies to heparin polysaccharides in humans have also been demonstrated (Shibata, 1998).

Unfortunately, following the above analyses, a preparation containing enough protein for N-terminal sequencing could not be obtained. This procedure would have allowed the relevant protein bands, as highlighted by Western blot, to be unequivocally determined as HXO.

Recently, Linder et al. (1999) failed to detect XO in human heart utilising rabbit anti-(HXO) pAbs. However, their studies were performed on whole heart tissue, in which low levels of XO will have produced only a small, and possibly undetectable, signal. Previously, the same group did find evidence for the presence of the heart enzyme in the form of low levels of XO mRNA (Saksela et al., 1998). In the present study, the hearts used were diseased. It is possible that disease leads to the expression of the enzyme, which may account for the inability to detect heart XO in previous studies.

The role of low activity HXO present in human heart still needs to be determined. The NADH oxidase activity of the enzyme remains intact and, therefore, so does the capability to produce ROS (see Section 1.2.2). Therefore, the damage attributed to XO during ischaemia/reperfusion may still be possible. It is arguable that although XO is present in heart, it is at such a low level as to have little consequence following an I/R event. However, the current study only concerns the presence of HXO in total heart, and not its exact location. It may be the case that the enzyme is localised in a single
cell type, thereby concentrating its effects. One outcome of ROS production by heart HXO may be to initiate a variety of intracellular signalling pathways. Such pathways include the mitogen-activated protein kinase (MAPK) sub-families. Activation of these subfamilies e.g. the stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs) by H$_2$O$_2$ has been demonstrated in perfused hearts (Sugden et al., 1997; Clerk et al., 1998a) and myocytes of rats (Clerk et al., 1998b) and the subsequent hypertropic response may lead to cell death through apoptosis. Therefore, the occurrence of the cardiac HXO has interesting ramifications given the oxidative stress the heart will be subjected to during I/R.

In conclusion, the immunoaffinity chromatography provides the most likely route to a one-step purification of the enzyme from tissue. Improvements will need to be made regarding the recovery of activity and the removal of the contaminating immunoglobulin. However, the use of a monoclonal antibody in future offers the potential to obtain pure enzyme.
Chapter 7

Final discussion


7. Final Conclusion

Since 1981 research into XO has been considerable following the publication of the I/R hypothesis by Granger et al. (1981). This has led to the implication of XO in a range of pathogenic conditions (McCord, 1985). Subsequently, the properties of the bovine milk, rat and chicken liver enzymes, have been well characterised. However, given the interspecies variability and apparent tissue-dependent activity of the enzyme, information gleaned from animal models cannot be extrapolated to humans. To date relatively little is known about the human enzyme. However, it does seem improbable that nature would generate an enzyme to produce ROS solely for damaging effects. To that end, the ability of XO to act as a source of superoxide and hydrogen peroxide, and the recent finding that the enzyme can generate NO (or peroxynitrite), implicates the enzyme in a potential bactericidal role (Harrison, 2002). However, XO also produces uric acid, a potential scavenger of ROS, therefore weakening the argument that XO is a purely destructive enzyme. In order to establish a function for the enzyme its precise tissue localisation and activity needs to be determined.

Only the liver and intestine are known to contain XO of high activity in humans, with XO mRNA also found to be highest in these tissues. Curiously, although abundant in milk, the enzyme from this source has surprisingly low activity toward traditional purine substrates, suggesting a tissue specific role for the enzyme. Given the potential for I/R damage during myocardial infarction, attention naturally turns to the heart, where reported XO activities range from high ((Wajner and Harkness, 1989), to low (Muxfeldt and Schaper, 1987; de Jong et al., 1990; Abadeh et al., 1993) to undetectable (Podzuweit et al., 1991; Eddy et al., 1987; Grum et al., 1989). The general consensus is that the heart enzyme is of low activity, reflected by the detection of XO protein in this tissue by the majority of workers employing
immunoaffinity techniques (Hellsten-Westling, 1993, Moriwaki et al., 1993, Abadeh et al., 1993), methods independent of enzyme activity. The present study has detailed the procedure by which XO can be purified from human tissue by immunoaffinity chromatography.

Recent improvements in the purification of human milk XO, i.e. removal of an initial stirring stage, inclusion of DTT in all buffers and omission of salicylate, have led to an increase in yield (Godber, 1998). Abadeh (1992) reported recovery of 18% of the total enzyme from breast milk, in the current study this has been increased to 56% typically, on average 22.1 mg of XO being purified from 1 litre of milk. Another modification was that the NaCl concentration of the pre-elution buffer was lowered from 0.1 M to 0.08 M. As a result, the purity of the enzyme on elution from the heparin column was improved, with an ideal PFR of 5.0 being achieved consistently, therefore removing the need for the anion exchange chromatography stage used by Sanders et al. (1997). A previous study into immunoaffinity purification of XO from human tissue used bovine milk XO for antibody purification (Price, 1997). The improvements detailed here allowed human milk XO to be purified in the amounts necessary not only to be used as the immunogen in order to generate polyclonal anti-(HXO) antibodies, but also to act as the ligand in their affinity purification.

The antibodies generated were not specific for the human form of the enzyme, indicating a high degree of homology between the bovine and human XO tertiary structures. Furthermore, it is safe to assume that if the antibody could detect both high (bovine milk) and low (human milk) activity forms of XO from different sources, it should do so for XO displaying a range of activities in the same species. Once affinity purified, the anti-(HXO) antibodies proved to be a versatile tool for the detection of XO in cell and tissue samples. A clue to possible role for XO was obtained from the immunolocalisation studies performed on cultured endothelial and epithelial cells (Rouquette et al., 1998; Appendix). There have been previous reports of a possible vesicular cytoplasmic occurrence internally
(Angermuller et al., 1987; Dikov et al., 1988). However, the punctate perinuclear occurrence of XO observed is a novel finding, which has more recently been confirmed in human endothelial and epithelial cells with the use of mouse monoclonal antibodies (Hoare, 2002). A possible explanation for this vesicular localisation is for storage of XO prior to export from the cell via a non-classical secretory pathway. Another exciting discovery was the asymmetrical external localisation of the enzyme on epithelial and endothelial cells (Rouquette et al., 1998; Appendix). Subsequently, XO has been detected on the outer surface of cultured bovine and porcine endothelial cells (Vickers et al., 1998) and at the luminal surface of rat liver sinusoidal cells (Frederiks et al., 1999). The logical explanation at this stage for such a discrete extracellular localisation is that XO is performing an intercellular signalling role, its ROS serving as signal transduction mediators.

The ultimate use for the antibody was in purification of XO from human tissue. IgG with very high avidity for XO will be present within the heterogeneous population of antibody. Elution conditions were therefore optimised in order to recover as active enzyme as possible. Incubation experiments using a selection of eluants (4 M urea, 3 M KSCN, 2 M NaSCN, 50 mM diethylamine, 2.8 M MgCl₂ and 0.01% SDS) were performed. Following extensive testing, diethylamine at 35 mM proved to be the most suitable eluant as it was effective at removing adsorbed antibody/enzyme, without irreparably damaging the columns. Also, time required for elution was shorter and volume less than required with the use of 4 M urea. A further consideration was that diethylamine will not N-terminally block proteins, allowing N-terminal sequencing to be performed by future investigators.

On elution, the activities of the pure milk enzyme used to test the immunoaffinity column were found to be 34%, 47% and 37% of the original xanthine, pterin and NADH oxidase activities, respectively. By immediate neutralisation and incubation of the eluted enzyme with FAD, these values were improved upon, increasing the xanthine, pterin and NADH oxidase activities to 50%, 53% and 74%, respectively. This is encouraging, but far from
ideal, tests on other eluants not included in the current study need to be performed before settling for diethylamine. Also, a gradient elution system could be employed to determine the most suitable concentration of eluant to elute the enzyme and return it in as active form as possible. Future work would also involve the generation of a specific anti-(HxO) monoclonal capable of removing the enzyme from tissue homogenate, but with an affinity allowing gentler elution conditions to be employed.

Human liver has been reported to contain XO of high activity (Krenitsky et al., 1986; Parks and Granger, 1986; Sarnesto et al., 1996) and its use allowed the efficacy of each stage of the purification to be monitored. Surprisingly, the XO activity of the crude homogenate appeared to increase during initial stages of purification, reasons proposed for this include the presence of an endogenous inhibitor, incomplete removal of endogenous purines, or quenching of the fluorescent signal by haemoglobin during the pterin assay. This observation in itself is worthy of further investigation.

The aim of the study was to use an immunoaffinity technique for tissue XO purification. However, heparin chromatography served as a useful parallel procedure to monitor activity loss on overnight circulation of the crude extract and diethylamine elution from the antibody column. Heparin mediated purification is not without its own drawbacks (Section 6.4) and the product yielded was not as pure as that obtained by immunoaffinity purification, as determined by SDS-PAGE. Although not attempted in the present study, future purifications could make use of both procedures.

The purified liver XO proved to be of high activity compared to milk. The activities obtained were 677 and 306 nmol isoxanthopterin/min/mg for the heparin and immunoaffinity purifications respectively. This constitutes a 45% recovery of activity on immunoaffinity chromatography compared to the heparin technique. Discrepancies between this and the 53% recovery obtained when purified milk enzyme was subjected to immunoaffinity
chromatography may be attributed to degradation by a mitochondrial protease of the type described by Saksela et al. (1998). The activity obtained via heparin chromatography compares favourably with the 1800 nmol urate/min/mg reported by Krenitsky et al. (1986) as pterin is oxidised slower than xanthine by XO (Beckman et al., 1989). Whilst the xanthine activity for milk enzyme is approximately four times faster than the pterin activity, Beckman and co-workers state that for human liver this could be nearer 8.5 fold, thus yielding a comparative xanthine activity of 5.75 μmol/min/mg. This value is very high, even by bovine standards and therefore future work would involve conclusively determining this xanthine:pterin activity ratio for each tissue.

Samesto et al. (1996), using a radioactivity assay in tandem with ELISA, similarly determined a high specific activity for human liver of 2.7 μmol/min/mg. Recently, values obtained from ELISA have been questioned, there may be a tendency for this to underestimate XO protein, thereby producing artificially high specific activities (Choudhury, 2001). The ELISA used for determining XO protein therefore requires further investigation.

Western blotting of the heparin purified liver sample identified a major band at 140 kDa, with a faint band at 155 kDa. The latter band was not detected in the immunoaffinity-purified sample, which may be attributable to further proteolysis during the extra day required for this procedure. However, a previous immunoaffinity purification of human liver, using a monoclonal antibody and 4 M urea as eluant, yielded fragmented enzyme that contained a monomeric band. In contrast, Samesto et al. (1996) failed to detect a band at 155 kDa in liver homogenates, reporting molecular weights of 143, 125 and 87 kDa on SDS-PAGE. The authors suggested that XO may be present in liver in a partially proteolysed form, however this is at odds with Moriwaki et al. (1993), who affinity purified XO from liver cytosol, yielding a monomeric 150 kDa band on SDS-PAGE. The mitochondrial protease described by Saksela and co-workers (1998) may again be the reason for the further degradation reported in the present study and warrants additional
investigation. Also, studies need to be performed to identify the optimum time for circulation of the crude homogenate on the column. If this stage can be shortened, the exposure of XO to endogenous proteases will also be reduced, perhaps resulting in detection of the monomeric 155 kDa band purified in the heparin procedure.

XO activity was not detected in whole tissue homogenate or heparin purified samples of human heart. However, very low activity enzyme was detected in immunoaffinity-purified samples, with a specific activity of 0.379 nmol isoxanthopterin/min/mg, equivalent to 0.28 μU/g wet weight. This low activity, even compared to the largely inactive human milk enzyme, suggests the XO to be endogenous to the heart, rather than present as a result of its release from other tissues. This is again at odds with reported high activity enzyme detected by Wajner and Harkness (1989) and explains why previous groups have been unable to detect XO in human heart (Eddy et al., 1987; Grum et al., 1989; Podzuweit et al., 1991). Full characterisation of human heart XO is still some way off, given that the whole tissue was found to contain only 445μg XO in total.

Hellsten-Westing (1993) suggested there to be differences between tissue XO activities due to the variety of tissue necropsies. Also, elevated expression and activity of XO has been demonstrated in failing rat hearts (de Jong et al., 2000) therefore, the pathology of the tissue examined also needs to be considered. The (patho-) physiological state (i.e. diseased or healthy), method of obtaining the heart (i.e. biopsy or post-mortem) may have an effect on the level of XO expression and activity. Further research is required to clarify the extent of these effects.

Western blots of immunoaffinity-purified heart XO indicated bands at 155, 140 and 90 kDa to be XO protein. These are largely consistent with band patterns obtained from liver XO purified by Krenitsky et al. (1986), Hellsten-Westing (1993), Saksele et al. (1999) and Sarmesto et al (1996), with the exception of a 125 kDa band in the latter study. Co-purifying
non-HXO protein was as a result of the failure to block unoccupied protein A sites on the matrix. Further experimentation would involve the optimisation of this procedure, perhaps with a protein A affinity chromatography stage prior to the immunoaffinity step, in order to produce a purer product.

Not addressed in the current study is the cellular distribution of heart XO. Immunolocalisation studies have indicated XO to be present in the endothelium of capillaries and small vessels in the heart (Hellsten-Westing, 1993). Enzyme located in heart endothelium has been confirmed by Moriwaki et al. (1993), but not by Linder et al. (1999). XO has also been detected in macrophages (Hellsten-Westing, 1993, de Jong et al., 2000) and mast cells (Hellsten-Westing, 1993). Clearly, establishing the cell type responsible for the XO activity detected in the present study would be advantageous and may further aid in elucidating the role of the enzyme. This was beyond the scope of the current thesis, however is certainly worthy of future research.

The amino acid sequences of the 'high' activity human liver and intestine enzymes have been shown to be essentially identical (Ichida et al., 1993; Saksela and Raivio, 1996; Yamamoto et al., 2001). Furthermore, the cDNA sequence of human mammary gland XO, a source of 'low' activity enzyme, has recently been found to be essentially identical to the other human cDNA sequences (Pearson, 2001). Sequencing the very low heart enzyme would be an interesting extension to these studies.

In summary, this work confirms earlier reports of very low activity XO present in human heart. This low activity, allied to the small amount of XO protein present, suggests the enzyme is unlikely to be responsible for the cardiac damage observed following I/R. Additional work might involve determining the molecular basis of this specific activity, the cellular and sub-cellular localisation of the enzyme and sequencing of the heart enzyme
cDNA. Unfortunately, the function of XO in human heart cannot be confirmed at this stage, however, its unique specific activity compared to human liver and human milk XO suggests a tissue-specific role for the enzyme.
Appendix
Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture

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Abstract

Subcellular localisation of xanthine oxidoreductase (XOR) was determined by indirect immunofluorescence using confocal microscopy in human endothelial and epithelial cell lines and in primary cultures of human umbilical vein endothelial cells. XOR was diffusely distributed throughout the cytoplasm but with higher intensity in the perinuclear region. In non-permeabilised cells, XOR was clearly seen to be asymmetrically located on the outer surface, showing, in many cases, a higher intensity on those faces apposed by closely neighbouring cells. Such specific distribution suggests a functional role for the enzyme in cell-cell interactions, possibly involving signalling via reactive oxygen species.

Key words: Xanthine oxidoreductase; Immunolocalization; Endothelial; Epithelial; Human

1. Introduction

Xanthine oxidoreductase (XOR) is a molybdenum-containing flavoenzyme that catalyses the hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid in the latter stages of purine catabolism [1]. In mammals, it occurs in two interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204) and xanthine oxidase (EC 1.1.3.22). Both forms of the enzyme can reduce molecular oxygen, although only the dehydrogenase form can reduce NAD, which acts as an electron acceptor. Reduction of oxygen leads to superoxide anion and hydrogen peroxide and it is the potential to generate these reactive oxygen species that has led to widespread interest in the enzyme as a pathogenic agent in many forms of ischaemia-reperfusion injury [2]. More recently, reactive oxygen species are being increasingly cited as intermediates in normal signal transduction pathways [3,4].

XOR is widely distributed, being particularly rich in mammalian epithelial cells and in capillary endothelium in a range of tissues [5,6]. While the enzyme is generally understood to be cytosolic, there have been very few published investigations of its precise subcellular localisation. Jarasch et al. [5] used both light and electron microscopic immunohistochemical procedures to show that XOR is located throughout the cytoplasm of bovine capillary endothelial cells. This was also found to be the case in rat liver endothelial cells [7,8]. In contrast, using immunoelectron microscopy, Ichikawa et al. [9] concluded that the enzyme was exclusively cytosolic with no significant association with intracellular organelles, including endoplasmic reticulum, Golgi apparatus, lysosomes or peroxisomes.

The apparent confusion over the subcellular location of XOR prompted us to investigate the situation in human cells. The human enzyme is of especial interest, particularly in view of questions regarding its anomalous characteristics and physiological role [10]. We have, accordingly, made use of confocal microscopy in immunolocalisation of the enzyme in human endothelial and epithelial cells in culture. We show here that XOR is present not only in the cytoplasm but also on the outer surface of all three cell types studied. Moreover, the extracellular enzyme shows a strongly polarised distribution, being in many cases concentrated on those surfaces closely apposed by neighbouring cells.

2. Materials and methods

2.1. Materials

RPMI 1640 culture medium was obtained from ICN, Costa Mesa, CA, USA. Penicillin, streptomycin and foetal calf serum (FCS) were from Life Technologies, Paisley, UK. Rabbit anti-TGN 46 antibody was a kind gift from Dr. George Banting (Department of Biochemistry, University of Bristol, UK). Rabbit anti-(bovine milk XOR) was from Chemicon International, Harrow, UK. All other reagents, unless otherwise stated, were from Sigma, Poole, UK.

2.2. Cell culture

EA-hy-926, a permanent endothelial cell line [11], was a gift from Dr. Andrew George, Imperial College School of Medicine, Hammersmith Hospital, London. The cells were routinely grown in an atmosphere of 5% CO\textsubscript{2}/95% air in 75-cm\textsuperscript{2} flasks at 37°C, as previously described [12]. Growth medium, RPMI 1640, containing 10% (v/v) FCS and penicillin/streptomycin [12], was changed every 3-4 days. The cells grew to form a confluent monolayer after approximately 7 days, exhibiting typical endothelial cell characteristics and were shown, by immunofluorescence (results not shown), to be positive for factor VIII, as reported by Edgell et al. [11]. HB4a is a human mammary epithelial cell line, conditionally immortalised by transfection with SV40 virus [13] and kindly donated to us by Dr T. Kamalati and Professor B. Gusterson of the Institute for Cancer Research, Royal Cancer Hospital, Sutton, UK. HB4a cells were routinely grown at 37°C in 75-cm\textsuperscript{2} culture flasks, in an atmosphere of 5% CO\textsubscript{2}/95% air as previously described [14]. Growth medium, RPMI 1640 containing 10% (v/v) FCS and other supplements [14], was changed every 3-4 days. Cells grew to confluence, forming a strict monolayer after 9 days, showing a characteristic 'crazy paving' appearance and stained strongly positive (results not shown) for the epithelial cell marker, cytokeratin [14].

Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical veins (kindly donated by the nursing staff of the Princess Anne Wing, Royal United Hospitals, Bath) and cultured essentially as described by Jaffe et al. [15].
2.3. Immunolabelling of cells and confocal microscopy

Cells were seeded (approx. 2 x 10^5 cells/ml) in four-chambered glass slides (Nunc Inc., Naperville, IL, USA), incubated for 24 h at 37°C and washed twice with pre-warmed PBS before fixing for 20 min at room temperature with 4% (w/v) formaldehyde in PBS. Cells were permeabilised by incubation with 0.1% (w/v) saponin in PBS for 45 min, then incubated with rabbit polyclonal anti-(human XOR) antibodies in PBS (0.02 mg/ml), containing 0.1% (w/v) saponin, 3% (w/v) normal goat serum and 1% (w/v) BSA, for 2 h at room temperature. The cells were washed three times with 0.1% (w/v) saponin in PBS before incubation, for 2 h at room temperature, with secondary antibody (FITC-conjugated goat anti-rabbit IgG (0.025 mg/ml, Jackson Immunoresearch Labs. Inc., West Grove, PA, USA)), diluted 1:100 in the same diluant as for the primary antibodies. The cells were then washed three times with PBS containing 0.1% (w/v) saponin, before removing the chambers from the slides prior to confocal microscopy.

Unpermeabilised cells were obtained and treated as above, except that saponin was omitted throughout. The permeabilised or unpermeabilised nature of the cells was confirmed by immunolabelling with rabbit anti-TGN 46 antibody, which is specific for the trans-Golgi network (results not shown) [16].

Images were collected on a confocal laser-scanning microscope (LSM 510, with either X40 1.30 NA or X63 1.40 NA apochromatic objective; Carl Zeiss, Welwyn Garden, UK). The 488 lines of an argon laser were used for excitation of FITC.

2.4. Assay for XOR enzymic activity

Cells were extracted and assayed for total (oxidase plus dehydrogenase) activity as previously described [14], using a sensitive fluorimetric procedure [17]. EA-hy-926 and HB4a cells contained 1-2 pmol isoxanthopterin/min/mg. Activity of HUVECs was below the lower limit of sensitivity of the assay (0.1 pmol isoxanthopterin/min/mg).

2.5. Heparin-Sepharose treatment of growth medium

A column (3.5 cm x 1.5 cm) of heparin-Sepharose (Sigma) was washed with appropriate growth medium (30 ml) lacking FCS. Growth medium (100 ml) containing FCS (10%) was then passed through the column and collected in a sterile container. The column was washed with 25 mM sodium phosphate buffer, pH 7.4, until a has reached a baseline level, and then with the same buffer containing 1 M NaCl. Protein-containing fraction (A_280) was assayed for XOR enzymic activity as described above.

2.6. Generation and affinity purification of rabbit polyclonal anti-(human XOR) antibodies

Antibodies were generated and affinity-purified as previously described [14]. Their specificity has been established by immunoprecipitation of XOR from HB4a cell extracts [14]. Incubation of HB4a cell extracts with the gel-bound specific antibodies removed 100% of XOR enzymic activity, while SDS-PAGE of the immunoprecipitate showed only the characteristic band of XOR, apart from bands attributable to the antibodies themselves [14].

3. Results

Three human cell types, including endothelial (EA-hy-926) and epithelial (HB4a) cell lines and primary endothelial (HUVEC) cells in culture, were studied using affinity-purified rabbit anti-(human XOR) antibodies (see Section 2). In all cases, immunolocalisation of XOR in permeabilised cells showed the enzyme to be diffusely distributed throughout the cytoplasm, although fluorescence in the perinuclear region was more intense (Fig. 1). Immunolocalisation of XOR in unpermeabilised cells clearly showed the presence of the enzyme on the outer cell surface, the distribution being localised to specific areas of the cell (Fig. 2). In several cases, XOR appeared to be concentrated on those parts of the surface that apposed or were extending towards neighbouring cells (Fig. 2C, arrows).

Use of commercially supplied rabbit anti-bovine milk
immunolabelling. No difference was apparent in the distribution or intensity of the fluorescence patterns between unpermeabilised cells grown in heparin-treated and untreated medium, nor was there any significant difference in total XOR activity in the cells, as assayed fluorimetrically. Results for EA-hy-926 cells are shown in Fig. 3C-F, in which the polarised distribution of the enzyme is again clearly seen. In the second control experiment, heparin-Sepharose beads (300 μl) were washed twice with PBS before incubation overnight, with continuous gentle agitation, either with growth medium (containing FCS), PBS or with PBS containing bovine XOR (10 μg/ml). Subsequent labelling with anti-XOR antibody showed clear immunofluorescence on the surface of the beads in the latter but not the former case (Fig. 3). Similar incubation of heparin beads with normal goat serum, used as a blocking agent in immunolabelling, also failed to lead to immunofluorescence on the surface of the beads.

4. Discussion

While reactive oxygen species are increasingly being considered as agents of signal transduction [3,4], their source is seldom clear, and XOR, with its capacity for generation of superoxide anion and hydrogen peroxide, is in many cases an attractive candidate [10]. The subcellular localisation of the enzyme is clearly relevant to its function and it is with

Fig. 3. Control experiments showing that cell surface XOR is not derived from growth medium. Heparin beads were incubated for 24 h with growth medium (A) or with PBS containing bovine XOR (B) (see Section 3). C, D and E, F show EA-hy-926 cells grown in medium (containing FCS), that has (E, F) or has not (C, D) been preabsorbed on a heparin-Sepharose column (see Section 2). Immunofluorescence (A-C, E) and DIC (D, F) images were obtained as described in Section 2. Magnification ×100; bar, 50 μm (A, B); ×400; bar, 20 μm (C-F).

If we accept that the extracellular XOR is indeed an endogenous enzyme, then the mechanisms of its secretion come into question. The classical secretory pathway of protein biosynthesis involves transfer from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane and depends upon the presence of a cleavable signal peptide [23]. Human XOR has no signal peptide [24,25] and is not known to be glycosylated, a consequence of the classical secretory pathway. However, increasing numbers of polypeptides with these characteristics, that are nevertheless secreted from both prokaryotic and eukaryotic cells, are being discovered [26] and it may well be that XOR is another such protein using a non-classical secretory pathway. In view of the relatively high affinity of XOR for heparin [18,19], it is interesting to note an earlier suggestion [27] that muscle L-14 lectin, exported by a non-classical pathway, would thus be separated from glycoconjugates, with which it interacted, until after its secretion. Similar considerations could apply to XOR, which may be expected to bind to cell surface glycosaminoglycans following secretion. It is noteworthy that incubation of EA-hy-926 cells with heparin, followed by washing, failed to significantly diminish the intensity of staining (results not shown), suggesting that other glycosaminoglycans may be involved.

Our results clearly show that XOR is not only present on the outer surface of cultured human endothelial and epithelial cells, but is asymmetrically distributed, in many cases appearing to be localised to surfaces apposed to those of closely neighbouring cells. This extracellular localisation and particularly its polarised nature strongly suggest a role for XOR in cell-cell interactions, possibly involving signalling via reactive oxygen species. We believe this to be an entirely novel concept worthy of detailed further investigations. Such investigations are, however, beyond the scope of the present study.

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source of reactive oxygen species that activate nuclear transcription factors, such as NF-κB [20].

XOR was clearly detected on the outer surface of unpermeabilised cells of all three human cell types studied. While extracellular localisation of XOR has previously been proposed in bovine aortic endothelial cells [21,22], our presently reported findings constitute the first detailed evidence of such a localisation in any cell type. In view of the potential importance of these results, it was necessary to eliminate the possibility that surface enzyme is derived from exogenous sources, such as, for example FCS in the growth media. Growth media did not contain levels of XOR above the limit of sensitivity of the fluorimetric assay. This, in itself, does not necessarily preclude the presence of lower levels of enzyme. However, XOR was not detected in the growth media following attempted concentration of the enzyme by chromatography on heparin-Sepharose, nor were any differences in immunolabelling detected when any of the three cell types was grown in preabsorbed medium. Moreover, growth medium for all three cells failed to show fluorescence labelling of heparin-Sepharose beads when incubated with the latter. Similar results were obtained with goat serum, routinely used as a blocking agent in immunolabelling. Finally, it is highly unlikely that cell surface XOR originated in lysed neighbouring cells, which are at low density early in their growth cycle and are essentially 100% viable.

If we examine the former in cultured human cells.

XOR is generally assumed to be a cytoplasmic enzyme, although its precise localisation is unclear, having been described as being both peroxisomal [7,8] and exclusively cytosolic [9]. In the permeabilised cells of the present study, XOR was seen to be generally distributed throughout the cytoplasm but with more intense staining in the perinuclear region. This latter localisation has not, to our knowledge, been suggested previously and has interesting implications concerning possible functions of the cytoplasmic enzyme. A perinuclear location would, for example, accord with a role for XOR as a...
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