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Scientific Research

Purification and Characterisation of Xanthine Oxidoreductases from Local Bovids in Malta

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Abstract

Xanthine oxidoreductase (XOR) is a molybdoflavoprotein mainly involved in purine catabolism. It exists in two forms, the oxidase (XO) and dehydrogenase (XDH) which are inter-convertible within mammalian cells. Although various researchers have reported the extraction of mammalian XOR, no extractions have yet been carried out in Malta and subsequently no characterizations are available. In this study, XOR was successfully purified from bovine, caprine and ovine milk through a multistep purification process involving both chemical and chromatographic techniques. The molecular weights of the native enzyme were found to be 295 kDa, 281 kDa and 275 kDa, representing the bovine, caprine and ovine XOR respectively. Western blot showed XOR to be represented on SDS-PAGE by a minimum of three major bands having molecular weights of 151 kDa, 131 kDa and 85 kDa. While all samples showed activity on native PAGE, spectrophotometric assays revealed the bovine XOR to be the most active. Surprisingly, the addition of NAD^+ to the assay mixture inhibited enzyme activity of the bovine and caprine XOR whereas the ovine XOR doubled its activity in response to NAD^+. The latter also showed a lower binding affinity to heparin. Following incubation with trypsin, XOR was irreversibly converted to its oxidase form in all samples as reflected by the observed increase in XO activity.

Keywords

Xanthine; Xanthine Dehydrogenase; Xanthine Oxidase; Milk; Bovid; Malta

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1. Introduction

Xanthine oxidoreductase (XOR) is a molybdenum hydroxylase which is able to oxidize a wide range of heterocyclic molecules including purines, pyridines and pterins [1] [2]. In addition to its well established role in purine catabolism, Vorbach *et al*. [3] proposed that XOR also contributes to the innate immune system due to the antioxidant properties of uric acid. Recent studies have also identified XOR as an antibacterial agent in milk [4] [5].

XOR exists in two forms, as xanthine dehydrogenase (XDH, EC 1.17.1.4) and xanthine oxidase (XO, EC 1.1.3.22). Although the xanthine dehydrogenase is the most abundant form *in vivo* [6], in mammalian cells it interconverts to the oxidase form either through oxidation of the thiol groups (reversible) or proteolytic cleavage (irreversible) [1]. XO and XDH mainly differ in the structure of the flavin adenine dinucleotide (FAD) domain which affects their choice of electron acceptors. Eukaryotic XOR is a dimeric enzyme made up of two identical subunits each acting autonomously. The monomer is composed of three interlinked domains which constitute an electron transfer pathway for the redox reactions catalyzed by the enzyme (**Figure 1**) [7].

Ball (1939) employed pancreatin for the extraction and partial purification of XOR [8]. Apart from degrading the caseins, pancreatin also cleaves XOR, irreversibly changing it to XO. In addition to pancreatin digestion, Gilbert and Bergel (1964) increased the yield through the use of butanol, EDTA and sodium salicylate, but this procedure reduced the final purity of the protein [9]. Waud *et al.* (1975) later demonstrated that proteolysis actually compromised the purity of the enzyme and they achieved better purification through a combination of butanol extraction, ammonium sulfate precipitation and chromatography [10]. The lowest A_{280}/A_{450} value (a measure of protein: flavin) of 4.1 was reported by Zikakis in 1979, together with yields of 21% which are 110% higher than any method published before 1979 [8]. To release XOR from its lipoprotein membrane, Zikakis described the use of a mild non-ionic detergent such as Triton X-100, in preference to the harsher lipolytic enzymes or butanol [8]. Nonetheless, studies carried out by Briley and Eisenthal (1975) show that complete dissociation of XOR to yield the free enzyme was better achieved through tryptic digestion [11]. Various chromatographic techniques have been attempted, including calcium phosphate [9], folate affinity [12] [13], Sephadex G-75, Sephacryl S-200, Sephadex G-200 and DEAE Sepharose CL-6B anionic exchange [8]. By 1993, XOR was discovered to have an intrinsic affinity to heparin [14], a property which was later exploited for its purification [15] through the development of heparin columns.

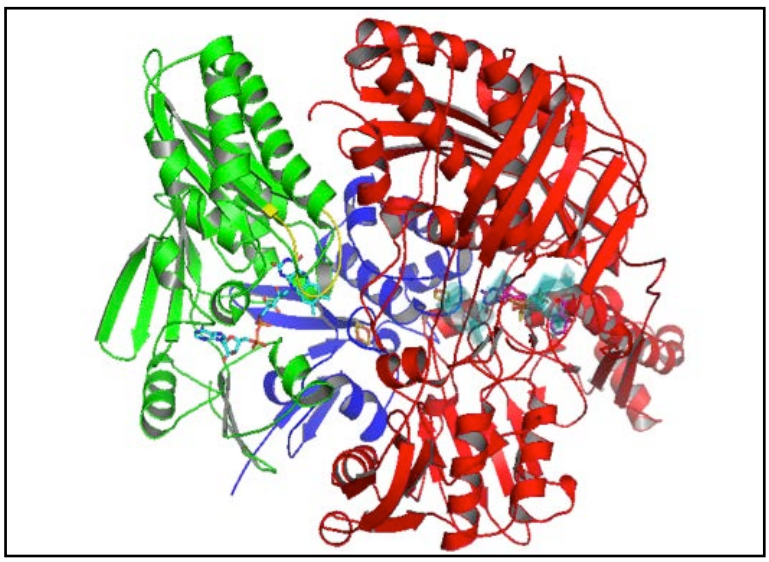

Figure 1. Structure of bovine xanthine oxidoreductase: The monomeric XOR depicting three individual chains corresponding to the FAD domain (green), iron-sulfur cluster domain (blue) and molybdenum domain (red). The biologically active molybdenum cofactor is shown in purple. The salicylate ion, also shown in purple is in proximity of molybdenum cofactor, acting as an active site protector. Two iron-sulfur clusters are depicted in yellow/orange. The yellow loop residing over the FAD cofactor is involved in the XDH-XO inter-conversion. Reproduced from PDB entry 1FIQ [7].

For more than a century, most of the studies on XOR were conducted on the bovine enzyme. It is only recently that scientists have gained interest in characterizing XOR from the milk of other mammalian sources [16]-[19]. Human XOR was first isolated, purified and characterized in 1986 from human liver [16]. In 2004, XOR was identified for the first time from both caprine [18] and ovine milk [19] in two independent studies, both of which showed the purified enzyme to be more active than the human XOR but substantially less active than the bovine enzyme [18] [19]. The aim of this study was to extract, purify and characterize XOR from domestic mammals local to the Maltese archipelago.

2. Materials and Methods

2.1. Chemicals

Unpasteurized bovine, caprine and ovine milk were donated by the Institute of Agribusiness (MCAST, Malta). General purpose chemicals together with cytochrome *c* from horse heart, trypsin (TPCK treated), Trizma base, NBT and xanthine were supplied by Sigma-Aldrich. Commercial bovine xanthine oxidase, EDTA sodium salt and dithiothreitol were from Roche while 2-N-Morpholinolethane-sulfonic acid, butanol and ammonium sulfate were purchased from VWR. Nicotinamide adenine dinucleotide (oxidised NAD), ECL Western blotting system, HiTrap Heparin HP column and Sephacryl^{TM} S-200 were from GE Healthcare and HRP-conjugated anti-xanthine oxidase rabbit polyclonal antibody and blocking reagent were from Pierce.

2.2. Protein Purification from Bovid Milk

The extraction methodology of bovid XOR is a modification of that reported by Sanders *et al*. (1997) who described the isolation of XOR from human milk [20]. EDTA, pH 8.0 was added to fresh unpasteurized bovid milk (2 L) to a final concentration of 1 mM and then centrifuged at 3,000 g for 30 min at 4˚C. All subsequent purification steps were carried out at 4˚C. The upper cream layer was collected and resuspended in an equal volume of 0.2 M K_2HPO_4 containing 1 mM EDTA, stirred for 2 hours, and the centrifugation step repeated. The resultant subnatant was decanted and collected after first perforating the upper solid layer. This subnatant was filtered through glass wool and 15% v/v *n*-butanol (chilled to −20˚C prior use) was slowly added to the filtrate whilst stirring. Stirring continued while ammonium sulfate (15% w/v) was added over a 45 min period. The suspension was centrifuged at 10,000 g for 30 min, the subnatant filtered through glass wool and 20% w/v ammonium sulfate was added over a 30 min period. The mixture was left for a further 45 min and then centrifuged at 10,000 g for 30 min. The upper "golden" layer was collected, resuspended in an equal volume of 20 mM MES pH 6.5 and dialyzed overnight against 5 L of the same buffer. The resultant dialysate was centrifuged at 15,000 g for 1 hour and the solution was clarified by filtration through a 0.2 µm nylon syringe filter. A heparin column was equilibrated with 30 mL of 20 mM MES buffer pH 6.5 and the resuspension applied to the column. It was washed with 50 mL of 20 mM MES pH 6.5, 0.03 M NaCl and XOR was eluted using 20 mM MES, pH 6.5, 0.4 M NaCl. The brown elution fractions were dialyzed against 5 L of 0.01 M sodium phosphate buffer, pH 7.5. Protein concentration was determined through A_{280} measurements and the BCA^{TM} protein assay (Pierce). Protein purity was assessed by 8% SDS-PAGE and molecular weight was determined by Sephacryl^{TM} S-200 gel filtration chromatography.

2.3. Preparation of the Dehydrogenase and Oxidase Forms of Xanthine Oxidoreductase

Recovery of the XDH form of the enzyme was ensured by the inclusion of 10 mM DTT at each step of the purification process. The xanthine oxidase form was prepared by incubating the XDH fractions collected from the affinity heparin column with 30 $\mu g \cdot mL^{-1}$ trypsin in 10 mM Na_2PO_4, pH 7.5 for 20 hours at 30˚C. DTT and trypsin were both removed from the protein by passing the sample through a second heparin column.

2.4. Western Blotting

Pure DTT-treated XDH samples were resolved by 15% SDS-PAGE and then electroblotted onto nitrocellulose membrane under semi-dry conditions using the LKB Multiphor II with a continuous transfer buffer system (Tris 48 mM, glycine 39 mM, SDS 0.0375% w/v, methanol 20% v/v) at 0.8 A/cm^2 for 70 minutes. The membrane was blocked overnight with blocking reagent, followed by three washes with freshly prepared TBS-T (TBS + 0.1%

™ Tween-20). The primary antibody was diluted 1:10,000 in TBS buffer. XDH protein was visualized by enhanced chemiluminescence.

2.5. Zymography for Xanthine Oxidase Activity Using 8% Native PAGE

The method used was a modification of that described by Zikakis (1979) and the activity stain solution consisted of 10 mM xanthine in 25 mM NaOH and 15 mM nitroblue tetrazolium (NBT) [8]. Prior to NBT addition, the pH of the solution was adjusted to 8.3 through the drop-wise addition of 1 M Tris-Cl pH 6.8

2.6. Spectrophotometric Assays for Xanthine Dehydrogenase Activity

Pure XOR fractions were assayed for enzyme activity using a DU7500 diode array spectrophotometer, (Beckmann). The assays performed were a modification of the method employed by Sanders *et al*. (1997). The oxidase activity was measured at 25˚C by monitoring the production of uric acid at a wavelength of 295 nm using an absorption coefficient of 9.6 $mM \cdot cm^{-1}$ [18] [20] [21]. The protein sample (50 µL) was added to working solution (950 µL) consisting of 100 µM xanthine solution in 0.05 M sodium phosphate buffer pH 7.5 using a 10 mM xanthine in 25 mM NaOH stock solution. Absorbance readings were recorded at 1-second intervals over a one minute period. The assays were repeated in the presence of 1 mM NAD^+.

In a separate experiment, XDH samples (1 mL) were incubated at 4˚C in the presence of excess FAD and then assayed for activity both in the presence and absence of NAD^+. The same samples were also analysed by the cytochrome *c* assay using a solution of cytochrome *c* consisting of 60 mg cytochrome *c* in 350 mL of potassium phosphate buffer pH 7.8. This cytochrome *c* solution (900 µL) was mixed with 50 µL of xanthine, NaOH (10 mM xanthine, 25 mM NaOH) and 50 µL of xanthine oxidase sample. Reaction rates were calculated by monitoring the reduction of cytochrome *c* at a wavelength of 550 nm at 25˚C, using an absorption coefficient of 21.0 $mM \cdot cm^{-1}$ [21].

3. Results

3.1. Enzyme Extraction and Purification

Purification of xanthine oxidoreductase from three different bovids local to the Maltese archipelago was carried out by standard methods. Bovine milk provided XOR of the highest specific activity and greatest yield while ovine milk gave the least (**Table 1**). Purified XOR from bovid milk exhibited multiple protein bands when analysed by SDS-PAGE (**Figure 2A**). The bands corresponding to XOR subunits were identified by immunoblot using an HRP conjugated anti-XOR rabbit polyclonal antibody and confirmed the molecular weights to be of 151.4 kDa, 131.8 kDa (**Figure 2B**). An 85.2 kDa fragment and a 64.6 kDa band, the latter present in the commercial bovine XOR sample, also showed a prominent affinity towards the XOR-specific antibody. Fainter bands, having molecular weights of 40.7 kDa and 19.5 kDa, present on SDS PAGE were not detected by Western blot.

3.2. Enzyme Purity and Molecular Weight Determination

Bovine, caprine and ovine XOR exhibited a protein to flavin ratio (PFR) of 4.88, 6.43 and 5.09 respectively after elution from the Hitrap™ heparin column. Following gel filtration chromatography, the PFR values recorded were 3.77, 6.27 and 4.52 respectively, reflecting a higher degree of purity. Aside from attaining a higher degree of purity, gel filtration was also used to determine the apparent molecular weight of the native XOR. The results obtained showed the local bovine, caprine and ovine XOR to have apparent molecular weights of 295 kDa, 281 kDa and 275 kDa respectively (**Figure 3**).

Andrews *et al*. report the enzyme's molecular weight to be 251 to 321 kDa [22], whereas Cheng *et al*. determined it to be between 225 and 370 kDa [23]. Most studies carried on bovine XOR state the enzyme's molecular weight as 275 [24], 283 [25] or 290 kDa [7]. XOR from other mammalian sources has been reported as having an apparent molecular weight of 300 kDa [18] [19] [26].

3.3. Enzyme Activity

While all samples showed activity when stained on native PAGE (**Figure 4**), spectrophotometric analysis re-

Table 1. Purification table for the extraction of Xanthine Oxidoreducctase from bovid milk. Assays were performed without addition of NAD^+.

Purification step		Volume (mL)	Protein		Enzyme Activity		% Yield
			Concentration ($mg \cdot mL^{-1}$)	Total (mg)	Specific activity (Units $mg^{-1} \times 10^3$)	Total Activity (Units)	
Fresh milk	Bovine	1, 775	26.82 ± 3.56	47,000.0	1.0 ± 0.1	47.00	100
	Caprine	2,800	23.63 ± 3.17	66,000.0	0.5 ± 0.2	35.00	100
	Ovine	2,800	50.68 ± 3.46	142,000.0	0.3 ± 0.2	42.60	100
n-butanol extraction	Bovine	62	6.89 ± 0.65	427.8	26.0 ± 0.6	11.10	24
	Caprine	222	8.24 ± 0.29	1820.4	1.0 ± 0.4	1.80	5
	Ovine	200	12.17 ± 0.13	2440.0	3.0 ± 0.3	7.30	17
Ammonium sulphate (20%)	Bovine	11	10.14 ± 0.45	111.1	43.0 ± 2.0	4.78	10
	Caprine	27	8.19 ± 0.50	139.3	13.0 ± 1.0	1.81	5
	Ovine	16	7.50 ± 0.35	120.0	4.0 ± 0.2	0.48	11
Heparin column	Bovine	5	1.45 ± 0.09	7.2	131.0 ± 6.0	0.94	2
	Caprine	4	4.49 ± 0.21	18.0	26.0 ± 2.0	0.47	1
	Ovine	5	3.61 ± 0.09	18.0	6.0 ± 1.0	0.11	0.3

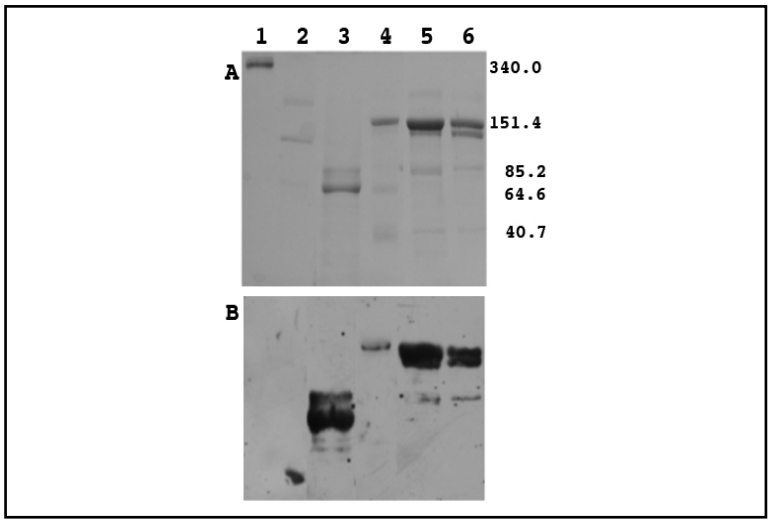

Figure 2. A: 8% SDS-PAGE: Lane 1: Non-reduced α-Macroglobulin, (3 µL, 340 kDa); Lane 2: Calibration standard proteins (5 µL, 170, 116.4, 85.2 kDa); Lane 3: Commercial XO (12.5 µg); Lanes 4 - 6: Sample from bovine, caprine and ovine milk respectively (5 µL). B: Western blot, same gel as in A, using polyclonal anti-XOR antibody.

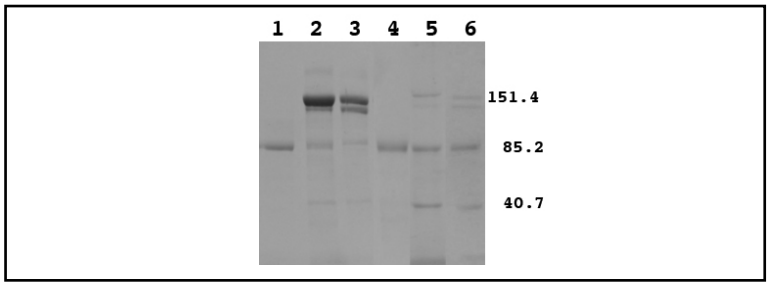

Figure 3. 8% SDS-PAGE of samples after affinity chromatography (lanes 1 to 3) and after gel filtration chromatography (lanes 4 to 6). Lanes 1 and 4; bovine, lanes 2 and 5; caprine, lanes 3 and 6; ovine.

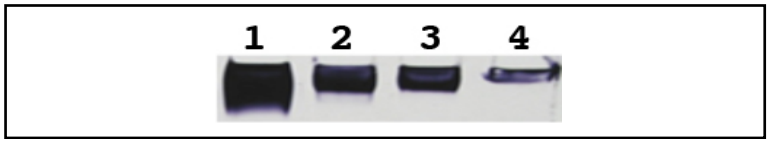

Figure 4. 8% Native PAGE stained for Xanthine Oxidase activity using NBT. Lane 1: Commercial XO (12.5 ug); Lanes 2 - 4: Samples from bovine, caprine and ovine preparations respectively (5 µL).

vealed the bovine XOR to be the most active sample and the ovine the least (**Table 2**). Each sample was analyzed in the presence and absence of NAD^+. NAD^+ is the preferred electron acceptor of XDH as opposed to XO which prefers O_2. In the presence of NAD^+, both the XDH and XO forms are expected to exhibit maximum catalytic activity [18]. However both bovine and caprine enzymes unlike the ovine sample, showed a decrease in uric acid production in the presence of NAD^+. The presence of excess FAD did not increase the reaction rate. Once again, the addition of NAD^+ was inhibitory to the overall reaction rate.

XOR samples were also analyzed for their ability to generate superoxide anions through the cytochrome *c* assay (**Table 3**). The change in A_{550} reflected successful reduction of cytochrome *c* by reactive oxygen species. The bovine XOR showed the highest activity and caprine the least. This is in agreement with the findings of Benboubetra and Baghiani (2004) who reported the ovine XOR to be more active than the caprine XOR, although both are significantly less active than the bovine enzyme.

Following tryptic digest, two major bands were seen on SDS-PAGE corresponding to molecular weights of 85.2 kDa and 72.0 kDa. A fainter band of 23.0 kDa was observed in the ovine sample. This pattern of bands was similar to that observed for the commercial bovine XOR (**Figure 5**), implying that the latter is also subject to proteolytic digestion. The trypsin-treated bovine and ovine enzymes showed a marked increase in oxidase activity, especially the bovine sample whose activity increased nine fold, indicating successful conversion of dehydrogenase to oxidase. Since the presence of FAD did not seem to increase the reaction rate, the observed rise in uric acid production was solely attributed to proteolytic digestion by trypsin.

4. Discussion

4.1. Protein Extraction and Purification

The extraction and purification of XOR from local bovid milk was achieved through chemical and chromatographic means. Samples from bovine and caprine milk showed a strong binding affinity to an immobilized heparin ligand, whereas ovine XOR did not. XOR has been reported to elute at low salt concentrations only when elution is carried out in the presence of alkaline buffers [14]. Given that all buffers employed in this study had a pH of 6.5, it appears that the ovine XOR has different chemical properties compared to the bovine and caprine counterparts. There is greater than 94% homology between the three bovid sequences (**Figure 6**). One major difference in the known sequences of XOR isolated from these sources is a deletion of 17 amino acids at position 1211 and an insertion of 12 amino acids at position 1266 in the ovine sequence relative to the others (bovine numbering).

Further research on ovine XOR may help to establish the nature of these differences; ovine XOR has only recently been purified [19] and there is very limited data on its structure. The latter would provide data on the relative external distribution of the lysine and arginine residues in the ovine XOR, which are responsible for the affinity of XOR to heparin [14].

Peptide bands corresponding to the subunits of XDH and XO (molecular weights 151.4 kDa and 131.8 kDa) were observed on SDS-PAGE gels and Western blots. The remaining faint bands, having molecular weights of 85.2 kDa, 40.7 kDa and 19.5 kDa are likely to be dissociated proteolytic fragments of XOR (**Figure 2**). Their presence even after gel filtration chromatography makes it unlikely that they are impurities. This is in agreement with the studies of Krentisky, Spector and Hall who report having achieved partial proteolytic fragmentation of the human liver XOR subunits on SDS-PAGE [16]. Cheng *et al*. (1988) showed that after protease-induced fragmentation, XO was composed of three globular domains, of molecular weight 85 to 100, 30 to 35 and 18 to 20 kDa [23]. Mangino and Brunner (1976), report that degradation of XOR into its subunits also occurs naturally due to endogenous proteases which are present in the milk itself and are co-isolated with XOR during its extraction, which accounts for the fragments obtained in this studies even in the apparent absence of

Table 2. Specific activity of XOR measured spectrophotometrically at 295 nm, using an absorption coefficient of 9.6 $mM \cdot cm^{-1}$. Samples were measured in the absence of additives, with NAD^+ or FAD only or with both NAD^+ and FAD. Each reaction was carried out in triplicate and an average value calculated, to ensure reproducibility. 1U is defined as the conversion of 1 µmole of xanthine into 1 µmole of uric acid per minute at 25˚C, pH 7.5. All activities are expressed as specific activities; per milligram of enzyme used. ND: not determined.

Sample	Specific activity (Units $mg^{-1} \times 100$)			
	Commercial XOR	Bovine XOR	Caprine XOR	Ovine XOR
No addition	77.0 ± 0. 4	13.1 ± 0.6 (125.0 ± 0.1)	2.6 ± 0.2	0.6 ± 0.1 (2.7 ± 0.1)
NAD^+	51.4 ± 0.2	8.5 ± 0.2	1.3 ± 0.1	1.1 ± 0.1
FAD	ND	12.0 ± 2.3	1.7 ± 1.0	0.5 ± 0.2
NAD^+/ FAD	ND	9.0 ± 2.7	1.0 ± 0.3	1.4 ± 0.1

Values shown in parenthesis represent enzyme activity after tryptic digest. Purified bovine XOR from a large scale extraction exhibited a specific activity of 104.0 ± 0.8.

Table 3. Cytochrome *c* assay. The reduction of cytochrome *c* by superoxide anions was monitored at a fixed wavelength of 550 nm, using an extinction coefficient of 21.0 $mM \cdot cm^{-1}$. This reduction is an indirect measure of XOR activity which produces reactive oxygen species under the reaction conditions used. 1 U is defined as the oxidation of 1 µmole of reduced cytochrome c per minute at 25˚C. Triplicate readings were recorded for each sample.

Sample	A550 min^{-1}	Specific activity ($Units \cdot mg^{-1}$)
Commercial bovine XOR	0.074	0.460 ± 0.002
Bovine XOR (large scale)	0.060	0.563 ± 0.003
Bovine XOR	0.035	0.435 ± 0.005
Caprine XOR	0.054	0.003 ± 0.001
Ovine XOR	0.022	0.005 ± 0.002

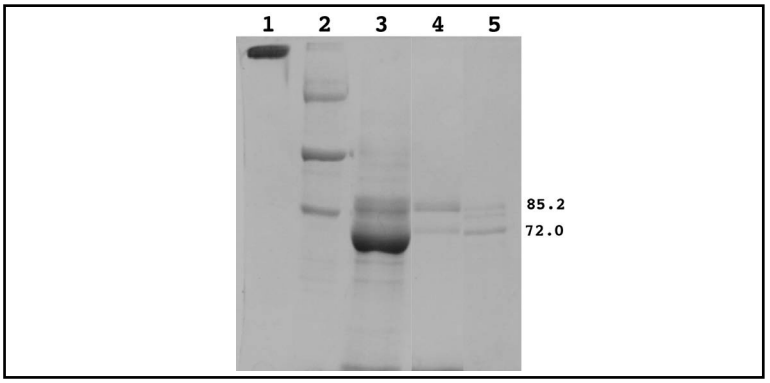

Figure 5. 8% SDS-PAGE of the tryptic digested samples. Lane 1: Non-reduced *α*-Macroglobulin (5 µL, 340 kDa); Lane 2: Calibration standard proteins (10 µL, 170, 116.4, 85.2 kDa); Lane 3: Commercial XO (12.5 µg); Lane 4: Bovine XO collected during elution (5 µg); Lane 5: Ovine XO collected during elution (5 µg).

proteolytic enzymes [23] [27]. Despite this, other studies report a single major band (150 kDa) after extraction and purification of XOR from unpasteurized bovine milk [18] [19]. It is likely that this is due to experimental factors including speed of purification away from contaminating proteases.

While proteolysis with trypsin does lead to a loss of the dehydrogenase activity due to structural changes near the FAD cofactor binding site, it has negligible effect on the activity of the oxidase form [19]. This retention of oxidase activity is due to the hydrophobic nature of the resultant peptidyl fragments that remain in close associa-

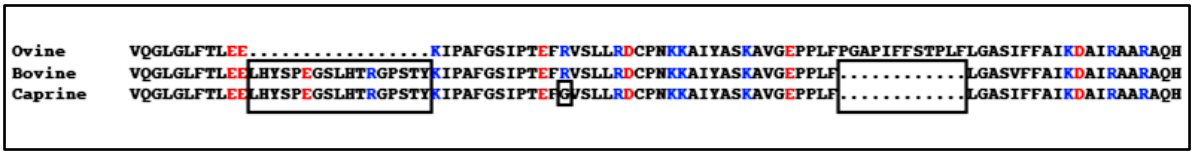

Figure 6. Multiple sequence alignment of Xanthine Dehydrogenases. Ovine (426223865), bovine (27806775) and caprine (119712145) obtained from the NCBI. Red and blue denotes negatively and positively charged residues respectively. Boxes highlight regions that are different from the ovine sequence and include a deletion of 17 amino acids at position 1211 and an insertion of 12 amino acids at position 1266 (ovine numbering).

tion with each other [20]. Indeed, Cheng *et al.* (1988) report having achieved a single band (151 ± 4 kDa) only on native PAGE electrophoresis [20]. In the absence of SDS, the hydrophobic nature of the enzyme maintains the individual subunits in close proximity to each other even in the presence of proteases, resulting in a slightly broader but single 150 kDa band on a native PAGE [8] [23].

4.2. Enzyme Activity

The final product is a mixture of both XO and XDH. In the absence of a suitable reducing agent, the majority of XOR is normally converted to its oxidase form [20] [28]. In this study, DTT was included during purification, so a high proportion of dehydrogenase was expected to be present in the final sample. Previous studies report that the sum of the oxidase and dehydrogenase activity may be determined by the addition of NAD^+ to the reaction mixture under aerobic conditions [19]-[21]. The results obtained in this study however, suggest that the reaction is actually inhibited by NAD^+ in both the bovine and caprine XOR. This is not the case for the ovine XOR. This decrease in reaction rate may be attributed to the production of NADH which is known to compete with NAD^+ for the FAD binding site. In the light of this, we postulate that the high proportion of dehydrogenase present in the sample led to a rapid build up of NADH production with consequent XDH inhibition. In the case of the ovine XOR, the addition of NAD^+ resulted in a two-fold increase in reaction rate. This differential activity exhibited by the ovine XOR may be related to either chemical or structural differences.

An alternative explanation to this differential activity in response to NAD^+ is due to the effect NAD^+ has on the rate limiting step of enzyme activity. Kinetic data carried out by Hunt and Massey [21] showed that xanthine oxidation occurs at a higher rate when catalyzed by XO rather than XDH. As such, the observed decrease in xanthine oxidation in the presence of NAD^+ can be explained in terms of the lower turnover number of XDH which relies on NAD^+ as its electron acceptor.

In agreement with literature data, the bovine XOR has the highest rate of xanthine oxidation both in the presence and absence of NAD^+. Caprine and ovine XOR showed a considerable lower activity possibly due to lower molybdenum content [18] [19] [29]. Although metal analysis was not part of this investigation, previous experiments report low molybdenum content in caprine and ovine XOR. Moreover it has been shown that activity of caprine and ovine XOR drastically increases after birth [18] [19]. Aside from reinforcing the antibacterial role of XOR in milk, this fact suggests that these mammals might have an intrinsic mechanism to incorporate molybdenum into XOR, so forming a fully functional XOR only when most required [19].

Addition of FAD slightly lowered the xanthine oxidation rate. This small decrease in activity may imply that the presence of excess unbound FAD was impeding enzyme activity, possibly due to alterations in pH. As there was no increase in reaction rate, it is likely that all the XOR enzymes were purified in their fully flavin-bound form. Komai, Massey and Palmer were the first to document the successful reconstitution of the flavoprotein in the presence of FAD and $(NH_4)_2SO_4$ at 25˚C. Nonetheless the same study also reports that unlike other flavoproteins, the flavin prosthetic group of XOR does not usually dissociate under normal purification conditions [30].

Whilst both XO and XDH produce uric acid as their final oxidation product, superoxide anions are mostly produced by XO in the absence of NADH. Although kinetic studies show that XDH can potentially produce more superoxide anions than XO via its NADH oxidase activity, in the absence of NADH, its interaction with O_2 is very slow, [21] especially if NAD^+ is present in the mixture. Although no NAD^+ was added to the cytochrome *c* assay, the samples analysed were all (except from the commercial XOR) mixtures of both XO and XDH, the latter being produced as a result of DTT addition during extraction. Consequently, a higher activity reading might not necessarily imply a more reactive enzyme, but possibly a higher proportion of XO in the sample.

4.3. Enzyme Yield

Bovine, caprine and ovine preparations yielded 18.6 mg (10 $mg \cdot L^{-1}$), 18.0 mg (6.8 $mg \cdot L^{-1}$) and 18.0 mg (6.18 $mg \cdot L^{-1}$) of enzyme respectively. This is similar to the findings reported by Sanders *et al.* (1997) who documented the recovery of 25 mg enzyme protein from 4 L of bovine milk, equivalent to 6.25 $mg \cdot L^{-1}$ [20]. A large scale preparation starting with 6 L of bovine milk gave a yield of 53 mg of protein which is equivalent to 8.83 $mg \cdot L^{-1}$.

4.4. Conclusion

XOR was successfully extracted and purified from local unpasteurized bovine, caprine and ovine milk, with the bovine, caprine and ovine preparations yielding 10 $mg \cdot L^{-1}$, 6.8 $mg \cdot L^{-1}$ and 6.18 $mg \cdot L^{-1}$ of active enzyme respectively. The dissociation of XOR from its membrane was achieved through butanol addition, without the involvement of proteolytic enzymes. Purified enzyme appeared as five major bands on SDS-PAGE of which only the first three fragments corresponding to molecular weights of 151.4 kDa, 131.8 kDa and 85.2 kDa were visible on a Western blot. All samples showed XOR activity when stained on native PAGE. Spectrophotometric analysis revealed the bovine enzyme to be the most active both in the presence and absence of NAD^+, followed by the caprine and then ovine sample. The addition of NAD^+ to the assay mixture was inhibitory and reduced the rate of uric acid production in the case of the bovine and caprine samples. Bovine XOR showed a nine fold increase in oxidase activity following tryptic digestion. No increase in the reaction rate was observed with the addition of excess FAD. When carried out on a large scale starting with 6 L of bovine milk, the extracted XOR showed an activity superior to the commercial bovine XOR for both spectrophotometric assays.

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