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Characterization of Human Colostral Xanthine Oxidase

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Human colostral xanthine oxidase was separated from the milk fat globule membrane and purified by standard techniques. Anion-exchange chromatography yielded XO activity peaks, which presented two active bands on disc-PAGE and ten active bands on isoelectric focusing. Cation-exchange chromatography yielded two XO activity peaks, which presented three active bands on disc-PAGE and nine active bands on isoelectric focusing. Analysis for isoenzymes confirmed the disc-PAGE results that suggested the two isolates contain both molecular size and charge isoenzymes. Subunit analysis found weights between 30 and 250 kDa. The two samples contained primarily acidic variants and the same cofactor ratios, namely, four atoms of iron, four atoms of sulfur, and one atom of molybdenum per molecule of flavin adenine dinucleotide. Treatment with neuraminidase indicated that *N*-acetylneuraminic acid was bound to human colostral XO and was not essential for catalytic activity.

Bovine milk is a rich source for xanthine oxidase (XO; E.C. 1.2.3.2), and a recent study showed that XO is the second most abundant protein on the bovine milk fat globule membrane (Burnier and Low, 1985). While one of the well-known functions of XO is the oxidation of purines, this does not merit its presence at such a high level. In fact, since its discovery over 100 years ago, investigators have attempted to explain the biological role and significance of the enzyme. Recent studies have implicated oxygen-derived free radicals to be the primary mediators of changes in vascular permeability associated with intestinal ischemia. Roy and McCord (1982) observed ischemia-induced conversion of xanthine dehydrogenase (E.C. 1.2.1.37) to XO, while Parks and Granger (1983) suggested the principle source of superoxide anions produced during intestinal ischemia to be XO. The superoxide radical may disproportionate to form hydrogen peroxide, which can, in turn, react with the superoxide in the presence of chelates to form the highly reactive hydroxyl radical (McCord and Day, 1978). The integrity of capillaries is presumed to be altered by this reactive radical via lipid peroxidation. In this process, hydroxyl radical removes an allylic hydrogen from a polyunsaturated fatty

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acid in the membrane of endothelial cells (Del Maestro et al., 1980). This could unleash a free-radical chain reaction, capable of damaging the membrane's integrity and perhaps causing cell death due to the lost membrane structure.

Bauer (1947) reported increased XO levels in the brain of mice with yellow fever, encephalitis, and lymphocytic choriomeningitis. High levels of the enzyme were also observed in the liver and polymorphonuclear leukocytes (PMNs) of mice during bacterial infection (Tubaro et al., 1980). Tubaro et al. (1980) demonstrate the role of XO in killing *Staphylococcus aureus* via PMNs by selectively inhibiting the enzyme. Ghezzi et al. (1984) suggested that interferon might account for the induction of XO and that it might augment enzyme activity. Reactive oxygen species are known to be involved in phagocyte-mediated microbial activity (Babior, 1978), in cellular cytotoxicity (Roder et al., 1982), and in tissue damage. Therefore, XO activity may play a role in the various biological activities of interferon, such as enhanced resistance to bacterial infections in mice treated with interferon inducers, enhanced activity of natural killer cells and monocytes, and depression of drug-metabolizing enzymes in the liver (Ghezzi et al., 1984). Furthermore, Topham et al. (1981) demonstrated that intestinal XO plays a major role in the absorption of dietary iron by promoting the oxidation and incorporation of iron into transferrin.

Prior to 1976, there was considerable debate whether human milk contains XO activity (Carr et al., 1975). Using radiochemical and polarographic assays, Zikakis et al. (1976) helped to settle this question. They assayed 59 human milk and colostrum samples and found XO activity in all samples with colostrum containing the highest activity. In view of the findings of Tubaro et al. (1980), the high level of XO in human colostrum may serve to protect the infant against infection from bacteria commonly present in milk. Furthermore, Zikakis et al. (1976) demonstrated conclusively that the enzyme in human milk is not of bacterial origin.

Despite these findings, some investigators remained skeptical of the existence of XO in human milk. The question was finally settled in 1983 when Zikakis et al. (1983) reported the isolation and partial purification of XO from human colostrum. Stability studies showed that human colostral XO has greater stability than bovine or caprine milk XO (Zikakis et al., 1983). Other differences found among human colostral XO and bovine and caprine XO included net charge, molecular weight, and amino acid composition.

In view of the importance of XO involvements in the numerous areas considered above and XO's implication in plasmalogen disease (Oster, 1971; Ross et al., 1973), there is a need to obtain undenatured, high-purity human XO for comparative studies. This information would permit differentiation between endogenous and exogenous XO in humans and would facilitate further studies into the biological role of XO. Partially purified human colostral XO preparations have been analyzed for kinetic parameters (Zikakis et al., 1983) and physical properties (Zikakis and McGinnis, 1987). The objective of this study was to obtain sufficient quantity of homogeneous human colostral XO and further characterize it as to its amino acid composition, ratio of cofactors, molecular weight, subunits, prosthetic groups, and isoenzymes.

MATERIALS AND METHODS

Materials. Frozen human colostrum (−20 °C) was obtained from the Mother's Milk Bank of the Wilmington Medical Center, Wilmington, DE. The following reagents were purchased from Sigma Chemical Co., St. Louis, MO: neuraminidase type V and VI, neotetrazolium chloride, flavin adenine dinucleotide (FAD), glycerol, fast green stain, iminodiacetic acid, 5-sulfosalicylic acid, anhydrous sodium sulfate, lauryl sulfate, dihydrate barium chloride, potassium chloride, ethylenediamine, and 2-mercaptoethanol. Tris(hydroxymethyl)aminomethane, sucrose, bromophenol blue, disodium ethylenediaminetetraacetic acid (EDTA), glycine, trichloroacetic acid (TCA), methanol, iron and molybdenum atomic absorption standards, ethyl alcohol, perchloric acid, and glacial acetic acid were purchased from Fisher Scientific Co., King of Prussia, PA. *N,N'*-Methylenebisacrylamide, riboflavin, Coomassie brilliant blue R-250, and xanthine were purchased from Eastman Organic Co., Rochester, NY. Acrylamide, protein assay dye concentrate, protein standard type I, and ammonium persulfate were obtained from BioRad Laboratories, Richmond, CA. *N,N,N',N'*-Tetraethylenediamine was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Sephacryl S-300, CM-Sephadex (C-50), DEAE-Sephacel, gel filtration molecular weight standards, electrophoresis calibration kit (used for all electrophoretic techniques described under Methods), and Pharmalyte 3-10 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Bactogel was purchased form Difco Laboratories, Detroit, MI. Other reagents and solvents were reagent grade. Glass distilled-deionized water was used throughout.

Methods. *Colostrum Preparation.* Upon arrival, colostrum was thawed and assayed for XO activity as given, vide infra. Samples containing greater than 1 ImU/mL were pooled and combined with 1% (v/v) of 20 mM sodium salicylate, 1% (w/v) EDTA, and 1 mg/100 mL trypsin inhibitor. After mixing, aliquots of 100–125 mL were stored at −20 °C for future isolations.

Isolation and Purification of XO. Frozen colostrum was thawed and combined with an equal volume of a buffer containing 8 mM sodium salicylate, 2.8 mM cysteine hydrochloride, and 0.2 M potassium phosphate (pH 6.8) and the resultant mixture incubated at 40 °C for 2 h with constant stirring. The sample was cooled to 4 °C, 1% (v/v) Triton X-100 was added, and the mixture was stirred for 15 min. All subsequent steps were carried out at 4 °C. Solid ammonium sulfate was added to a concentration of 20% (w/v), and the mixture was centrifuged at 12000*g* for 30 min. The upper fat layer was discarded and the supernatant filtered through glass wool. Ammonium sulfate (14%, w/v) was added to the filtrate; the mixture was stirred for 30 min and then centrifuged at 12000*g* for 90 min. The supernatant was discarded and the precipitate dissolved in 0.13 mM EDTA (pH 6.8). The dissolved precipitate was immediately dialyzed against three 1-L volumes of 0.13 mM EDTA changed after 1 and 2 h and removed after 3.5 h of total dialysis time. The retentate was combined with an equal volume of hydroxylapatite suspension [119.4 mg/mL, prepared according to Tiselius (1956)] and allowed to equilibrate overnight. The suspension was centrifuged at 12000*g* for 10 min and the supernatant assayed for XO activity. If XO activity was greater than 1 ImU/mL, more hydroxylapatite was added and the mixture was recentrifuged. XO was eluted stepwise from hydroxylapatite by successive batch washings with a series of potassium phosphate buffers (0.05, 0.1, 0.2, 0.3, and 0.4 M, pH 6.8, three washes with each buffer). All washes containing active XO were pooled and concentrated to approximately 8 mL by either ultrafiltration (using an Amicon XM-100A membrane) or reverse dialysis.

The concentrated XO preparation from the hydroxylapatite step was applied to a Sephacryl S-300 column (2.6

× 100 cm) previously calibrated with molecular weight standards, vide supra, and equilibrated with 0.1 M pyrophosphate buffer (pH 7.1) and eluted with the same buffer. The fractions collected were analyzed for both protein and XO activity, and those constituting the central portion of the major peak of activity were pooled and concentrated by ultrafiltration. The preparation was dialyzed against 0.005 M pyrophosphate buffer (pH 8.6) and the dialysate applied to either an anion- (DEAE-Sephacel) or a cation- (CM-Sephadex) exchange column (1.6 × 20 cm) equilibrated with 0.005 M pyrophosphate buffer, pH 8.6. Following application of the sample, the column was washed with four column volumes of 0.005 M pyrophosphate buffer, pH 8.6. The adsorbed XO was eluted with a 0.005–0.1 M pyrophosphate buffer gradient (pH 8.6) with a flow rate of 0.4 mL/min. Both columns were run as described above, except in the case of the cation-exchange column where the pH of the pyrophosphate buffer was 7.2. The 5-mL pregradient and gradient fractions were monitored for protein and XO activities. Major peak fractions were pooled and concentrated by ultrafiltration or reverse dialysis.

XO Activity. Colostrum samples were assayed for XO activity polarographically according to the method of Zikakis and Treece (1971) using an Yellow Springs Model 53 Biological Oxygen Monitor, and activity obtained in microliters of O_2 per milliliter per minute was converted to international units (IU) by the method of Zikakis (1979). Purified XO preparations were assayed on a Gilford Model 250 spectrophotometer, and activity was determined in international units (Avis et al., 1955, 1956; Zikakis and Silver, 1984).

Discontinuous Polyacrylamide Gel Electrophoresis (disc-PAGE). disc-PAGE was performed with various amounts of sample containing 0.05–0.1 mg of protein on a Buchler 18-tube polyanalyst. The system consisted of a 6.0% T, 2.6% C separating gel (T = total mass of acrylamide/volume of gel solution and C = mass of bisacrylamide/total mass of acrylamide) and a 3.2% T, 2.0% C stacking gel, and the samples were separated by the method described by Ornstein (1964). Gels to be stained for protein were first fixed in 12.5% TCA, then stained with 0.04% Coomassie blue in 3.5% perchloric acid, and destained with 10% methanol in 7.5% acetic acid. Gels to be stained for XO activity were placed directly into a sodium containing 2.8 mM neotetrazolium chloride in 10 mM xanthine, similar to the method of Zikakis (1981). Total protein concentration in the samples was determined by the Bio-Rad method (1979) using bovine serum albumin as the standard.

SDS Electrophoresis. Subunit molecular weights were determined by SDS electrophoresis according to the method of Weber and Osborne (1969) on 6.0% T, 2.6% C gels containing 2% SDS in 0.1 M sodium phosphate buffer (pH 7.0). Samples containing 20–50 μg of protein were run at 16 °C and 8 mA per gel for 6 h; the running electrode buffer was 0.05 M sodium phosphate (pH 7.0). Gels were fixed in a solution containing 40% methanol in 7% acetic acid and stained for protein as described under the disc-PAGE section above. Subunit molecular weights of XO were determined from a standard curve (relative mobility vs. log molecular weight, MW) using the following standard proteins: thyroglobulin (MW 330 000), ferritin (MW 220 000 and 18 500), albumin (MW 67 000), catalase (MW 60 000), and lactate dehydrogenase (MW 36 000).

Isoelectric Focusing (IEF). The IEF method described in Hoeffer Scientific Instruments Co. Manual (1983) was employed to determine the apparent isoelectric points of XO. Sample size ranged between 25 and 50 μg of protein and the 6.0% T, 2.7% C acrylamide gels contained pH 3–10 ampholyte. Gels to be stained for protein were fixed in 5% sulfosalicylic acid and 15% TCA and then stained with 0.1% fast green in a mixture of acetic acid–ethanol–water (10:30:60, v/v/v). Gels destined for XO activity localization were stained as described under the disc-PAGE section above. The p*I*s were obtained from a standard curve (pH vs. distance from the cathode) with the following standard proteins: amyloglucosidase (p*I* 3.50), soybean trypsin inhibitor (p*I* 4.55), β-lactoglobulin (p*I* 5.20), bovine and human carbonic anhydrase B (p*I* 5.85 and 6.55, respectively), horse myoglobin acidic and basic bands (p*I* 6.85 and 7.35, respectively), lentil lectin acidic, middle, and basic bands (p*I* 8.15, 8.45, and 8.65, respectively), and trypsinogen (p*I* 9.30).

Molecular Weight. The MW of XO was determined by gel filtration chromatography on a calibrated Sephacryl S-300 column and estimated from a standard curve of the following standard proteins: 7 mg/mL albumin (MW 67 000), 2 mg/mL aldolase (MW 158 000), 5 mg/mL catalase (MW 232 000), 0.5 mg/mL ferritin (MW 440 000), and 2 mg/mL thyroglobulin (MW 669 000).

Cofactor Ratio. The concentrations of iron and molybdenum were determined according to the procedure outlined in the Perkin-Elmer graphite furnace atomic absorption spectrophotometer assembly manual (Perkin-Elmer, 1982). Pyrolytically coated graphite tubes were used for the analyses. Total FAD was determined spectrophotometrically by the method of Yagi (1962). The blank (0.1 M pyrophosphate buffer, pH 7.1), standards (9.1, 18.3, 45.7, and 91.3 ppm FAD), and unknown samples were incubated at 38 °C overnight in triplicate. Absorbance was measured at 450 nm and the total FAD determined from the following equation (Yagi, 1962) FAD = $10/(E/0.144)$ μg/mL, where E is the absorbance at 450 nm. To ensure that all observed absorbance was from FAD, the enzyme preparation was decomposed. FAD was hydrolyzed to flavin mononucleotide by adding TCA to obtain a final concentration of 10%.

Sulfur was determined by combining the methods of Hanson (1976) and Tabatabai (1974). Briefly, saturated magnesium nitrate solution was added to the samples and to a standard containing L-methionine prior to ashing in an initially cold muffle furnace. The cooled ash was acidified with 3 N HCl and combined with a bactogel solution. Total sulfur was measured from a standard curve.

Determination of Prosthetic Groups. The presence of *N*-acetylneuraminic acid (NANA) was determined by treating XO samples with types V and VI neuraminidase (Emmelot and Bos, 1965). Six units of neuraminidase in 0.05M Tris–maleate buffer (pH 5.3) was combined with XO samples and the resultant mixture incubated for 1 h at 37 °C to release any NANA present. XO samples not treated with neuraminidase and a protein standard were isoelectric focused as described before.

Amino Acid Analysis. The amino acid composition of XO samples was determined according to the method of Lee (1974) using a Durrum Model D-500 high-pressure amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110 °C for 24 h under nitrogen. Tryptophan and cysteine are hydrolyzed under these conditions and were not determined (Lee, 1974).

Isoenzyme Analysis. The isoenzymes present in XO preparations were analyzed by the procedure of Hedrick and Smith (1968) using disc-PAGE. The gel concentration was varied between 5 and 10% by diluting a stock acrylamide solution (30% T, 2.6% C) to prevent any changes

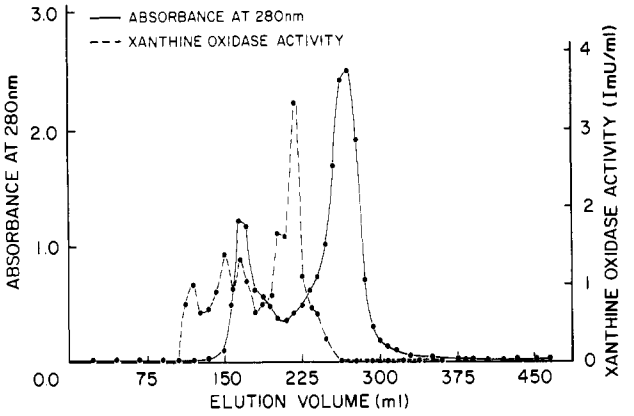

Figure 1. Elution profile from the Sephacryl S-300 superfine column. Xanthine oxidase was eluted with 0.1 M pyrophosphate buffer (pH 7.1) at a flow rate of 0.5 mL/min in 7.5-mL fractions.

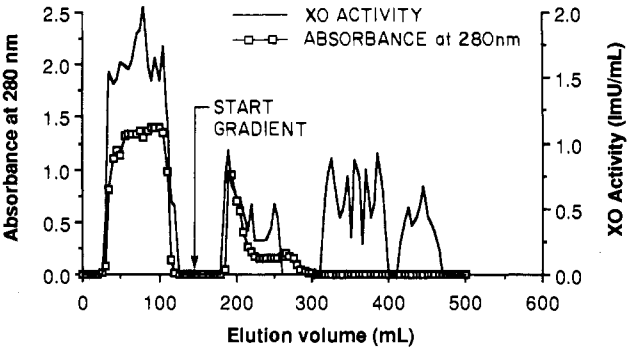

Figure 2. DEAE-Sephacel elution profile. Xanthine oxidase was eluted from the first 150 mL with 5 mM pyrophosphate buffer (pH 8.6). After 150 mL, a linear salt gradient from 5 to 100 mM pyrophosphate buffer (pH 8.6) was run at a flow rate of 0.3 mL/min in 5-mL fractions.

in bisacrylamide ratio that may have affected the sieving characteristics of the gel. Duplicate samples were run simultaneously with one gel stained for protein and its duplicate gel stained for XO activity.

RESULTS

Enzyme Purification. Figure 1 is a typical elution profile from the gel filtration column showing two protein peaks and four XO activity peaks. Rechromatographing the fractions under the second or third XO activity peak (using the same column) resulted in an elution profile with a peak distribution similar to the one in Figure 1. Since the fourth peak of XO activity contained a higher specific activity than the other three peaks, its fractions were pooled, dialyzed, and concentrated until the sample conductivity was equal to the conductivity of 5 mM pyrophosphate (pH 8.6 for the anion- and 7.2 for the cation-exchange columns) before being subjected to anion- and cation-exchange chromatography. The gradient anion-exchange chromatography of XO on DEAE-Sephacel (XO_{DEAE}) yielded one protein peak and ten XO activity peaks (Figure 2). Most of the XO sample applied to the DEAE column did not bind to the column and was detected in the pregradient wash before the gradient was initiated. disc-PAGE analysis of the XO_{DEAE} preparation gave ten protein bands containing XO activity with p*I* values between 4.4 and 7.3 (Table I).

The XO fraction that did not bind to the DEAE column was dialyzed and concentrated as before and applied to the cation-exchange CM-Sephadex column. Non-XO proteins in this fraction bound to the column while XO eluted in the pregradient wash and resolved into two broad protein peaks both of which contained XO activity. Since the total XO activity eluting in this region corresponded so closely to the amount of activity applied to the column, a gradient was not deemed necessary. Fractions containing XO activity from the CM-Sephadex (XO_{CM}) were concentrated and analyzed by disc-PAGE. This preparation resolved three protein bands, all containing XO activity. IEF analysis of the XO_{CM} preparation gave nine protein bands containing XO activity with p*I* values between 3.7 and 8.2 (Table I). Upon disc-PAGE, both the XO_{DEAE} and XO_{CM} preparations exhibited a similar enzymatically active protein band near the top of the gels.

Table I. Characteristics of the CM-Sephadex Purified XO (XO_{cm}) and DEAE-Sephacel Purified XO (XO_{DEAE})

characteristic	XO_{CM}	% of total[a]	XO_{DEAE}	% of total[a]
isoelectric	3.7		4.4	
points of	3.8		4.5	
enzymatically	4.0		6.2	
active bands[b]	4.2		6.5	
	4.4		6.6	
	5.0		6.8	
	5.4		7.0	
	8.1		7.1	
	8.2		7.2	
			7.3	
subunit MW[c]	249005	54.6	245573	9.7
	209572	30.1	215062, 199291	39.2, 45.5
	30129	15.3	30707	5.6

[a] Percentages were calculated from peak areas obtained from densitometer scans. [b] Obtained by isoelectric focusing. [c] Obtained by SDS electrophoresis

Table II. Cofactor Ratios of Xanthine Oxidase from Anionic (XO_{DEAE}) and Cationic (XO_{cm}) Exchange Chromatography

cofactor	XO_{cm}[a]	ratio[b]	XO_{DEAE}[a]	ratio[b]
FAD	109.0	1.00	26.2	1.00
iron	439.0	4.03	105.6	4.03
sulfur	448.0	4.11	100.1	3.52
molybdenum	98.1	0.90	21.0	0.80

[a] Concentration in nanomoles per milliliter. [b] Ratios are based on moles per milliliter of FAD equivalent to 1.

Molecular Weights. Fractions under the four peaks containing XO activity (which were obtained from gel filtration chromatography; see Figure 1) were pooled and concentrated. Their molecular weights were determined by applying the concentrated sample to a standardized Sephacryl S-300 column. The resulting weights for the four resulting enzymatically active peaks were 171, 296, 1530, and 2650 kDa. Since rechromatographing fractions under any of these isolates gave a similar elution profile (as in Figure 1), the enzyme was aggregating, a fairly common characteristic for membrane-bound proteins containing glycosylated moieties (Dunn and Maddy, 1976). Table I contains the subunit molecular weights of XO_{DEAE} and XO_{CM} as determined by SDS electrophoresis. As can be seen from data in Table I, XO_{DEAE} had three subunits and XO_{CM} four. The molecular weights of three subunits were similar in both preparations, but their quantitative distributions were different.

Cofactor Ratio. Table II lists the cofactor ratios for the XO_{DEAE} and XO_{CM} preparations. As it is customary, cofactor ratios for the milk enzyme are expressed with FAD assigned the stoichiometric coefficient of 1. The cofactors found for human colostral XO were approximately four atoms of iron, four atoms of sulfur, and one atom of molybdenum per molecule of FAD. The differences in nanomoles of cofactor per milliliter of XO_{CM} and XO_{DEAE}

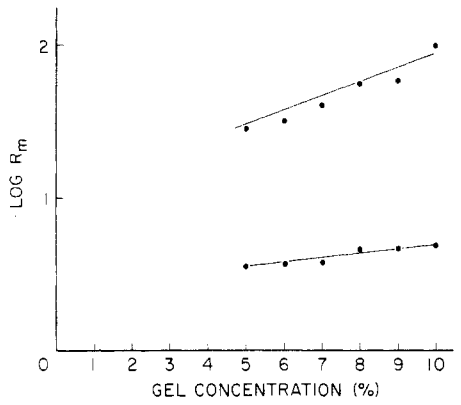

Figure 3. Isoenzyme analysis of xanthine oxidase from DEAE-Sephacel column. The system consisted of separating gels ranging from 5% T (left) to 10% T (right) with a constant 2.6% C, stacking gels (3.2% T, 2% C), and a Tris–glycine electrode buffer (pH 8.3). The upper line corresponds to the top bands on the gels while the lower line corresponds to the bottom bands.

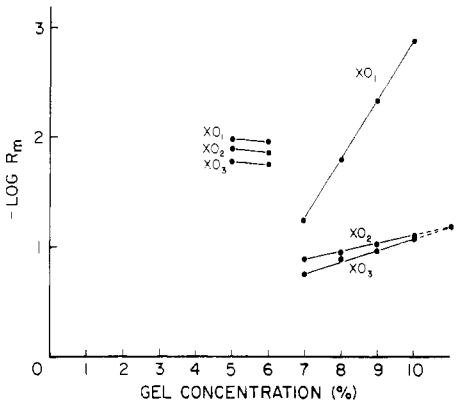

Figure 4. Isoenzyme analysis of xanthine oxidase from the CM-Sephadex column. The system consisted of separating gels ranging from 5% T (left) to 10% T (right) with constant 2.6% C stacking gels (3.2% T, 2% C), and a Tris–glycine electrode buffer (pH 8.3). XO_1 corresponds to the top bands, and XO_3 corresponds to the bottoms bands observed on the gels.

reflect the differences in the concentrations of the assayed samples.

Prosthetic Groups. Treatment of the XO_{DEAE} preparation with neuraminidase caused the p*I* values of the enzyme to shift from 6.82 to 7.00 in the acidic region and from 8.00 to 8.28 in the basic region. Similarly, neuraminidase-treated XO_{CM} shifted the p*I* values from 4.51 to 4.57 in the acidic region and from 8.79 to 8.95 in the basic region. The shift of the p*I* in the basic direction resulting from the action of neuraminidase demonstrates that NANA was ketosidically bound to XO_{DEAE} and XO_{CM} preparations (Cook and Stoddart, 1973) and suggests that human colostral XO is a glycoprotein.

Isoenzyme Analysis. The isoenzyme analysis of XO_{DEAE} is shown graphically in Figure 3. The nonparallel lines in the graph indicate that the two enzyme forms present differ in both their molecular size and charge (Hedrick and Smith, 1968). Figure 4 shows the isoenzyme analysis results for the XO_{CM} preparation. The family of lines is indicative of species with the same molecular size but different charges (Hedrick and Smith, 1968).

Amino Acid Composition. Table III contains the amino acid composition for human, bovine, and caprine XO. With a few exceptions, the amino acid composition of bovine and caprine XO is similar. XO from the three species contains high quantities of aspartic and glutamic acids. In bovine and caprine XO, these amino acids represent 19.1% and 19.8%, respectively, of the total amino acid composition. Furthermore, these two residues are highest (27.2%) in human XO.

DISCUSSION

Gel filtration chromatography of XO yielded four peaks containing XO activity with two peaks eluting before blue dextran (MW 5000) on a calibrated column. Gel filtration estimates the MW and the Stokes' radii of unknown proteins (Dunn and Maddy, 1976). In principle, separation occurs by molecular sieving, with relative elution positions being determined by relative particle dimensions. However, membrane-bound proteins (such as XO) have a tendency to aggregate into asymmetrical complexes (Dunn and Maddy, 1976). Furthermore, Katzman (1972) found that proteins aggregate while moving through a column. Our gel filtration results support the aggregation concept for membrane-bound proteins.

Since XO is such a large and dynamic enzyme, it is not a surprise that neither anion- nor cation-exchange chromatography fully bound the protein. In ion-exchange chromatography, kinetic processes such as transport of species in the void volume between the resin particles and exchange of ions at the fixed groups within the resin are as significant as longitudinal transport of solute-containing eluent through the column (Rothbart, 1973). In the case of XO where the subunits both aggregate and dissociate during chromatography, the sorption–desorption kinetics tend toward desorption since the subunit–subunit interactions are more attractive than the subunit–resin particle interactions. Thus, the problems encountered with anion-exchange chromatography make this technique less useful for fractionating membrane-bound proteins. The addition of a cation-exchange column to the purification procedure yielded more information.

On the basis of IEF results, the XO_{CM} was pure with respect to charge and consistent with the nine active variants observed on IEF. The majority of the bands corresponded to acidic forms of XO, but the binding patterns differed, an observation reported previously (Sullivan et al., 1982). According to these authors, when a protein is analyzed by IEF, it may migrate through an environment of successively lower pH values. This influences the ionization and the net charge of the protein. Since XO contains a high percentage of amino acids that can exhibit more than two pK_a's, the human enzyme can interconvert from charged form to charged form, and thus the ionization (and therefore the IEF) pattern will also change for the same sample.

Acidic forms of bovine milk XO were first isolated in 1939 (Ball, 1939). These various forms were assumed to be aggregation products of the bovine enzyme with membrane sialoglycoproteins (Sullivan et al., 1982). The numerous variants may reflect the presence of desulfo XO or may confirm the existence of immunologically and catalytically distinct enzymes (Krenitsky and Tuttle, 1978).

The negatively charged amino acids (aspartic and glutamic acids) comprised 27.2% of the total amino acid content of human XO, which is about 8% higher than the amount present in either the bovine or the caprine enzymes. Thus, on the basis of amino acid analysis, human colostral XO is indeed different from either the bovine or the caprine milk enzyme. This high concentration of negatively charged amino acids also partially explains the predominance of acidic variants on IEF for both XO_{CM} and XO_{DEAE}.

The cofactors observed for XO were approximately four atoms of iron, four atoms of sulfur, and one atom of molybdenum per molecule of FAD. This seems to agree with

Table III. Amino Acid Composition of Purified Xanthine Oxidase from Human Colostrum and Bovine and Caprine Milk

amino acid[a]	amino acid content, mol %		
	human[b]	bovine[c]	caprine[d]
aspartic acid	13.3	8.6	9.1
threonine	5.8	7.0	6.7
serine	9.1	6.5	5.4
glutamic acid	14.6	10.0	10.7
proline	4.3	5.5	6.6
glycine	7.8	8.2	8.7
alanine	6.8	7.6	7.7
valine	3.2	6.8	6.8
methionine	0.69	2.2	2.0
isoleucine	4.5	4.8	5.1
leucine	11.8	8.9	9.3
tyrosine	2.5	2.4	2.3
phenylalanine	3.6	4.9	5.0
lysine	7.8	6.9	7.0
histidine	4.2	2.3	2.5
arginine	3.4	4.7	5.0

[a] Ammonia, tryptophan, and cysteine were not included in the calculations. [b] This study, purified by anion-exchange chromatography. [c] Nelson and Handler (1968). [d] Zikakis et al. (1983).

the values reported for bovine milk XO where the Mo:Fe:S:FAD = 1:4:4:1 (Palmer and Massey, 1968). For XO_{CMI} more sulfur was present than iron while for XO_{DEAE} the reverse was observed. In the latter case, desulfo XO may have formed by spontaneous release of sulfur during purification.

Our results suggest that removal of NANA by neuraminidase did not affect the catalytic activity of human XO. In most animal tissues, bound sialic acid is mainly associated with proteins and NANA is most commonly found in higher vertebrates (Ng and Dain, 1976). Moreover, sialic acid is a constituent of many serum glycoproteins and possesses a relatively strong carboxyl group. Thus, the presence of NANA would contribute to the net negative charge of XO in a detectable manner. This would explain the shift to more basic p*I* observed when XO was treated with neuraminidase. Montgomery (1972) reported that sialic acid is mainly responsible for the electrophoretic microheterogeneity of glycoproteins, which also explains the numerous acidic variants observed for XO_{CM} and XO_{DEAE}.

Covalent modification of ionizable amino acid side chains can produce charge isoenzymes possessing distinctive electrophoretic mobilities, and modifications that affect nonprotein components may also lead to heterogeneity. The variation in the carbohydrate moieties of XO may have caused additional heterogeneity to be exhibited by the active enzyme.

Thus, human colostral XO is a heterogeneous enzyme differing from the bovine milk enzyme in amino acid composition, the presence of NANA, the presence of both molecular size and charge isoenzymes, and both tne number and the size of the subunits. This suggests that endogenous and dietary XO in humans can be differentiated on the above basis and thus facilitate the evaluation of questions on the origin of XO in cardiovascular and other diseases.

Registry No. XO, 9002-17-9.

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