Detection of xanthine oxidase and immunologically related proteins in fractions from bovine mammary tissue and milk after electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate

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A solid-phase immunoassay was used to detect xanthine oxidase in fractions from bovine mammary glands after electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Under these conditions the major proportion of xanthine oxidase in either mammary tissue or milk could be recovered as a protein of mol.wt. 150 000. In mammary tissue approx. 80% of the enzyme was in a soluble form and the remainder was accounted for in either 'mitochondrial' or microsomal fractions after tissue homogenization and fractionation. Affinity chromatography of either detergent-solubilized microsomal membranes or postmicrosomal supernatants on immobilized antibody to xanthine oxidase yielded a single protein that cross-reacted with antibody to the enzyme. In milk, presumptive degradation products of the enzyme were detected in minor quantities with mol.wts. of 43 000 in the whey fraction and 90 000 in fat-globule membrane. Only the undegraded enzyme was present in the skim-milk membrane fraction. Xanthine oxidase is therefore synthesized and secreted as a protein with a monomeric mol.wt. of 150 000 and is not subjected to extensive proteolytic degradation during the storage of milk in mammary alveoli. The significance of the results is discussed in relation to the overall protein composition of the membranes of milk-fat globules and skim milk.

The membrane surrounding fat droplets in milk has received increasing attention in recent years as a source of antigens derived from the actively secreting epithelial cells of mammary tissue (Ceriani *et al.*, 1980; Freudenstein *et al.*, 1979; Mather *et al.*, 1980; for reviews, see Anderson & Cawston, 1975; Patton & Keenan, 1975). A major reason for this interest is the convenience with which large amounts of apically derived membrane-bound protein can be isolated, purified and characterized.

Membrane, however, is isolated from milk that is stored in the mammary gland for various times before milking, generally up to 12 h in the case of bovine milk. The possibility has therefore been raised that at least some of the proteins of fat-globule membrane are the products of proteolysis, occurring either during or after secretion (Mangino & Brunner, 1977a). Proteinases have been identified both in the skim-milk phase and in association with the fat-globule membrane itself (Shahani *et al.*, 1973; Mangino & Brunner, 1977a; Eigel *et al.*, 1979).

Mangino & Brunner (1977a) raised the poss-

ibility that a significant proportion of the protein of fat-globule membrane is composed of xanthine oxidase (EC 1.2.3.2) and proteolytic breakdown products of the enzyme. This suggestion was based on the observation that their purified xanthine oxidase preparation on storage was hydrolysed to a series of peptides with apparent molecular weights similar to the major proteins of fat-globule membrane. The amino acid contents of both purified xanthine oxidase (Bray & Malmström, 1964; Hart *et al.*, 1970; Mangino & Brunner, 1977a,b) and unfractionated fat-globule membrane (Kobylka & Carraway, 1972; Mangino & Brunner, 1977b) are also similar, possibly suggesting a common identity.

Previous identification of xanthine oxidase has emphasized the isolation of enzymically active protein, although various inactive forms of the enzyme have also been described, some of which appear to be present *in vivo* (for a summary and discussion of this, see Bray, 1975). Apart from affinity-purified material (Edmondson *et al.*, 1972), purified preparations of xanthine oxidase contain variable proportions of non-functional enzyme.

To determine the relationship, if any, between

Abbreviations used: SDS, sodium dodecyl sulphate.

xanthine oxidase (either functional or non-functional, undegraded or proteolytically cleaved) and the other major proteins of fat-globule membrane we have purified xanthine oxidase and prepared specific antibody to the enzyme. This antibody has been used to identify immunologically related peptides in fractions of mammary tissue, fat-globule membrane and skim milk, irrespective of biological activity.

Materials and methods

Materials

6-Amino-n-caproic acid, ammonium persulphate, aprotinin, mercaptoethanol, *p*-nitrophenyl phosphate, phenylmethanesulphonyl fluoride, *NNN'*N'-tetramethylethylenediamine, Tris, xanthine, trypsin type IX (EC 3.4.21.4), chymotrypsin type I (EC 3.4.21.1), papain type IV (EC 3.4.22.2) and bovine serum albumin (fraction V; 96–99% albumin) were all from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals A–B (Uppsala, Sweden) and goat anti-(rabbit immunoglobulin G) conjugated with alkaline phosphatase (EC 3.1.3.1) was obtained from Miles Laboratories Inc. (Elkhart, IN, U.S.A.). Electrophoresis-grade acrylamide, *NN'*-methylenebisacrylamide, Coomassie Brilliant Blue R-250, Triton X-100, SDS and the molecular-weight standards myosin (EC 3.6.1.3), β -galactosidase (EC 3.2.1.23), phosphorylase *b* (EC 2.4.1.1), bovine serum albumin, ovalbumin, carbonic anhydrase (EC 4.2.1.1), soya-bean trypsin inhibitor and lysozyme (EC 3.2.1.17) were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Methods

Assays. Xanthine oxidase was assayed spectrophotometrically by following the oxidation of xanthine at 293 nm. Assay conditions were as described by Mather *et al.* (1980) with the omission of iodinitrotetrazolium.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Immunological detection of antigens after electrophoresis in SDS/polyacrylamide gels. Xanthine oxidase and immunologically related peptides were detected after electrophoresis in SDS/polyacrylamide gels by a solid-phase assay based on the procedure of Lutz *et al.* (1979).

Samples of SDS-solubilized protein were separated by electrophoresis in slabs of SDS/polyacrylamide as described previously (Mather *et al.*, 1980). After electrophoresis longitudinal strips (1.5 mm $\times$ 6 mm $\times$ 85 mm) containing the separated proteins were cut from the slab with 120-mm-long microtome blades. The strips were rinsed in water for 30 min at room temperature to remove excess SDS and then sliced laterally with an electric gel slicer (Bio-Rad model 195; Bio-Rad Laboratories).

Adjacent sections (2 mm total thickness) were then placed in 100 μ l portions of 0.1 M-NaHCO₃/Na₂CO₃ buffer, pH 9.4, containing 0.02% (w/v) NaN₃ and held in the plastic cuvettes of a Cuvette-Pak™ (Gilford Instruments, Oberlin, OH, U.S.A.). [Other plastic plates, such as the 96-well Immunolon™ plates (Dynatech Laboratories Inc., Alexandria, VA, U.S.A.), designed for solid-phase immunoassays can be used instead of the Gilford cuvettes, provided that a suitable spectrophotometer/reader is available to measure the absorbance of the solutions in the plates after colour development.] The slices were incubated at 4°C in a humidity chamber for 36 h, during which time antigen diffused from the gel and bound to the surface of the cuvette. Binding of protein to the plastic is assumed to involve primarily hydrophobic interactions [for discussion of this see Engvall (1980)]. The cuvettes were then washed four times with a mixture of 0.15 M-NaCl and 0.05% (v/v) Triton X-100 principally to remove excess SDS from the immobilized protein.

The presence of antigen was detected by adding to each well 100 μ l of specific antibody to xanthine oxidase previously diluted in 'serum dilution buffer' [0.15 M-NaCl containing 50 mM-Tris/HCl, pH 7.5, 0.02% (w/v) NaN₃, 0.05% (v/v) Triton X-100 and bovine serum albumin (0.1%, w/v)]. The samples were incubated at room temperature for 2 h in a humidity chamber and the cuvettes were subsequently washed three times with Tris-buffered saline [10 mM-Tris/HCl, pH 7.5, containing 2.68 mM-KCl, 137 mM-NaCl and bovine serum albumin (0.1%, w/v)].

Portions (100 μ l) of an alkaline phosphatase-conjugated goat antibody to rabbit immunoglobulin G, previously diluted in the above 'serum dilution buffer', were then added to each cuvette. After incubating for 2 h at room temperature in a humidity chamber the cuvettes were finally washed three times with Tris-buffered saline.

Bound alkaline phosphatase was detected by adding 0.3 ml portions of 2.5 mM-*p*-nitrophenyl phosphate in 50 mM-glycine buffer adjusted to pH 10.5 with NaOH. The cuvettes were incubated at room temperature and the absorbance of the solutions measured at 405 nm in a Gilford EIA Processor/Reader (model PR-50) at convenient time intervals (generally 0.5–4 h). Cuvettes were then always further incubated overnight to allow the detection of minor quantities of antigen.

Preparation of membrane fractions from mammary tissue and milk. Mammary tissue was obtained from three Holstein cows at slaughter at the Beltsville Agricultural Research Center abattoir (Beltsville, MD, U.S.A.). All three cows were in good lactational standing and were free of mastitis as determined by the absence of bacteria in aseptic

milk samples taken at monthly intervals before slaughter.

Mammary tissue was homogenized and fractionated by the method of Farrell (1980) with or without the inclusion of dithiothreitol in the homogenization buffer. Fresh tissue was used within 1 h of slaughter. Four fractions were obtained: 'nuclear', 'mitochondrial' and microsomal membrane fractions and a postmicrosomal supernatant. In three of the four preparations proteinase inhibitors were included in the homogenization medium, namely, aprotinin (approx. 25 kallikrein inhibitor units/ml), 0.1 mM-phenylmethanesulphonyl fluoride and 1 mM-6-amino-n-caproic acid.

Fat-globule membrane and skim-milk membranes were prepared from small samples of milk (approx. 200 ml/sample). Milk from three cows was combined and separated into cream and skim-milk fractions in the presence of the above proteinase inhibitors. The cream fraction was washed three times (Mather, 1978) and the membrane released from the fat globules by alternately freezing and thawing the samples a minimum of three times. Fat-globule membrane was then collected from the 'buttermilk' by centrifugation (Mather, 1978) and samples of membrane and the supernatant were retained. The skim-milk phase was separated by centrifugation into a casein sediment, an overlying skim-milk membrane fraction and a clear yellowish whey supernatant (Plantz & Patton, 1973). The casein sediment was discarded and the skim-milk membranes and whey fractions were retained for further analysis. The whole procedure was repeated five times (total of 15 cows). In addition fat-globule membrane was prepared five times without proteinase inhibitors as previously described (Mather, 1978).

Preparation of specific affinity media for xanthine oxidase. Xanthine oxidase was purified from washed milk-fat globules by extraction with butanol-1-ol, precipitation with $(\text{NH}_4)_2\text{SO}_4$, separation by preparative electrofocusing and chromatography on concanavalin A-agarose. Rabbit antibodies to the enzyme were prepared and purified by fractionation of the immune serum on xanthine oxidase covalently linked to Sepharose 4B. Full details of these procedures will be published separately (Sullivan *et al.*, 1981).

Affinity-purified antibody to xanthine oxidase (15 mg) was coupled to 3 g (dry wt.) of CNBr-activated Sepharose 4B by the method recommended by Pharmacia Inc., and stabilized with 0.025% (w/v) glutaraldehyde as described by Kowal & Parsons (1980). Before use the immunoadsorbent was washed with 75 ml of 0.1 M-sodium borate buffer adjusted to pH 8.0 with HCl and containing 1.0 M-NaCl and then with 75 ml of 3 M-sodium thiocyanate containing 0.1 M-Tris/HCl, pH 7.7. This

washing cycle was repeated two more times and the immunoadsorbent was finally equilibrated in 50 mM-Tris/HCl, pH 7.5, containing 0.2 M-NaCl.

Results

Solid-phase immunoassay of xanthine oxidase and peptides of xanthine oxidase

An initial study was performed to ensure that denaturation of the protein samples with SDS did not inhibit the subsequent binding of antibody to either xanthine oxidase or proteolytically cleaved fragments of the enzyme. Purified xanthine oxidase was hydrolysed with trypsin, chymotrypsin or papain and the samples were separated by SDS/polyacrylamide electrophoresis. The ability of antibody to bind to the separated fragments was then determined with the solid-phase immunoassay described in the Materials and methods section (Fig. 1). A single peak of activity coinciding with the major band of protein was obtained with the purified enzyme (Fig. 1a). Proteolysis gave rise to a series of peptides, most of which also reacted with antibody to xanthine oxidase as shown by the accompanying peaks of alkaline phosphatase activity (Figs. 1b, 1c and 1d). Specific antibody to xanthine oxidase therefore bound to the enzyme and fragments of the protein even after treatment with SDS. This experiment also shows that the antibody raised in rabbits to intact enzyme recognized many determinants on xanthine oxidase and was not specific to a limited, but highly immunogenic, region of the protein.

Detection of xanthine oxidase and immunologically related proteins in mammary tissue

When bovine mammary tissue was homogenized and fractionated (total of four preparations) xanthine oxidase activity was detected in the separated fractions as follows: 'nuclear' fraction, 0%; 'mitochondrial' fraction, $5.65 \pm 1.65\%$ (mean $\pm$ S.D.); microsomal fraction, $10.35 \pm 6.30\%$; postmicrosomal supernatant, $84.0 \pm 7.80\%$. (These values are expressed as the percentage of recoverable activity; recovery of activity from the total homogenate ranged from 50 to 70%.) Storage at -20°C and freeze-thawing of the fractions increased the overall amount of oxidase activity as observed in other tissues (Stirpe & Della Corte, 1969). The relative amounts of enzyme activity in each fraction, however, were not altered significantly.

All fractions were subsequently analysed for the presence of xanthine oxidase by solid-phase immunoassay after electrophoresis in SDS/polyacrylamide gels. No xanthine oxidase or immunologically related proteins were detected in the 'nuclear' fraction, confirming the above activity data. The 'mitochondrial' fraction contained a cross-reactive protein with a mol.wt. of approx.

Fig. 1. Proteolysis of purified xanthine oxidase
Purified xanthine oxidase (44 μ g) was incubated in a final volume of 100 μ l with 0.2 M-Tris/HCl, pH 7.5, containing 10 μ g of proteinase. The mixtures were incubated at 37°C for 20 min and the reactions stopped by the addition of 100 μ l of 0.125 M-Tris/HCl, pH 6.8, containing 8% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) mercaptoethanol. The solutions were heated at 100°C for 5 min and cooled on ice. Portions (50 μ l) were separated by electrophoresis in 10% (w/v) polyacrylamide gels containing SDS and antigen was detected with affinity-

30000, but no protein with a mol.wt. of 150000 similar to undegraded xanthine oxidase was detectable. In contrast, both the microsomal membrane fraction and postmicrosomal supernatant contained proteins with mol.wts. approx. 150000 that were cross-reactive with antibody to xanthine oxidase. In addition, the postmicrosomal supernatant appeared to contain an immunologically related protein of higher molecular weight (greater than 200000).

In all the above experiments with tissue samples, however, long incubation times with *p*-nitrophenyl phosphate were required (i.e., 12 h at 37°C) to detect xanthine oxidase and immunologically related proteins. Proteins from both the microsomal membrane fraction and the postmicrosomal supernatant were therefore separated by affinity chromatography on an immunoabsorbent consisting of specific antibody to xanthine oxidase coupled to Sepharose 4B. This served to concentrate and purify immunologically related proteins in sufficient quantity for further analysis. The results clearly showed that the only major protein in either the samples of microsomal membrane or the cell cytosol that was bound by and eluted from the immunoabsorbent had the same molecular weight as that of xanthine oxidase isolated from fat-globule membrane (Fig. 2). That this major protein eluted from the column cross-reacted with specific antibody to xanthine oxidase was confirmed by solid-phase immunoassay (results not shown). No other immunologically related proteins were detected.

Detection of xanthine oxidase and immunologically related proteins in fractions of milk

Xanthine oxidase activity has been detected previously in skim milk, the skim-milk-membrane fraction and whey. After disrupting washed fat-globules the enzyme is distributed in an approximately equal ratio between the soluble phase and fat-globule membrane (Briley & Eisenthal, 1974; Mather *et al.*, 1977). All these fractions were therefore analysed by the solid-phase immunoassay discussed above for xanthine oxidase.

In all samples the major protein that cross-reacted with specific antibody to xanthine oxidase had mol.wt. approx. 150000 and co-migrated with purified xanthine oxidase (see Fig. 3 for examples of fat-globule membrane, skim-milk membrane and whey supernatant; other fractions not shown).

purified antibody to xanthine oxidase (0.47 μ g of antibody in each cuvette). The cuvettes were incubated with phosphatase substrate for 1 h at room temperature. (a) Untreated purified enzyme; (b) trypsin-treated enzyme; (c) chymotrypsin-treated enzyme; (d) papain-treated enzyme. Arrowheads indicate the dye front for each gel.

Fig. 2. Analysis by electrophoresis in SDS/polyacrylamide gels of fractions from mammary tissue before and after affinity chromatography on immobilized antibody to xanthine oxidase

Microsomal membranes (5 mg/ml final concentration) were extracted with 0.5% (v/v) Triton X-100 as described for fat-globule membrane (Mather *et al.*, 1980). The soluble extract (25 ml) was diluted 1:1 with 0.1 M-Tris/HCl, pH 7.5, containing 0.4 M-NaCl and recycled over antibody to xanthine oxidase covalently linked to Sepharose 4B overnight at 4°C. The immunoadsorbent was subsequently washed with 150 ml of 50 mM-Tris/HCl, pH 7.5, containing 0.2 M-NaCl and specifically bound protein eluted with 50 ml of 3 M-sodium thiocyanate in 0.1 M-Tris/HCl, pH 7.5. Postmicrosomal supernatants were treated similarly but were not extracted with Triton X-100. Fractions were analysed by electrophoresis in 8% (w/v) polyacrylamide gels containing SDS. 1, Microsomal membrane; 2, postmicrosomal supernatant; 3, peak fraction after affinity chromatography of detergent-solubilized microsomal membrane; 4, peak fraction after affinity chromatography of post-microsomal supernatant; 5, milk-fat-globule membrane. Molecular-weight markers were as follows: (a) myosin; (b) xanthine oxidase; (c) β -galactosidase; (d) phosphorylase *b*; (e) bovine serum albumin; (f) ovalbumin; (g) carbonic anhydrase. The gels shown were stained with Coomassie Brilliant Blue R250.

Additionally a cross-reactive protein of mol.wt. 90000 was present in eight of the ten samples of fat-globule membrane (component X in Fig. 3a) and a cross-reactive protein of mol.wt. 43000 was present in three of the five whey samples analysed (component Y in Fig. 3b). All five skim-milk membrane fractions, however, contained only undegraded xanthine oxidase (Fig. 3c).

Fig. 3. Analysis by solid-phase immunoassay of samples of fat-globule membrane, whey and skim-milk membrane

Fractions (20 μ g of protein) were separated by electrophoresis in 10% (w/v) polyacrylamide gels containing SDS and assayed by solid-phase immunoassay using 1.12 μ g of antibody/cuvette and incubating with phosphatase substrate for either 2 h (membrane fractions) or 4 h (whey fractions). Representative samples are shown for: (a) fat-globule membrane; (b), whey; (c) skim-milk membrane. Arrowheads indicate the dye front for each gel.

Discussion

The analysis of fat-globule membrane from bovine milk by electrophoresis in SDS/polyacrylamide gels

results in the separation of seven or eight major zones of protein (Anderson & Cawston, 1975; Patton & Keenan, 1975). That one of these major zones is comprised, at least in part, of xanthine oxidase was realized by Mangino & Brunner (1977a) and Mather *et al.* (1977) and is confirmed by the present study (Fig. 3a). The lack of cross-reactivity of specific antibody to xanthine oxidase with any of the other major proteins of fat-globule membrane supports the contention that these proteins are immunologically distinct (Freudenstein *et al.*, 1979; Ceriani *et al.*, 1980; Mather *et al.*, 1980; Franke *et al.*, 1981) and does not agree with the proposals of Mangino & Brunner (1977a) discussed in the Introduction. The present study also shows that the inactive 'demolybdo' form of xanthine oxidase seen *in vivo* (Hart *et al.*, 1970) most likely has the same monomeric molecular weight as fully active enzyme and is not in the form of proteolytically cleaved fragments either free or bound together in a stoichiometric complex (Waud *et al.*, 1975; Nagler & Vartanyan, 1976). The traces of presumptive degradation products of xanthine oxidase seen in whey and fat-globule membrane fractions (components X and Y, Fig. 3) may be the same as the fragments with mol.wts. 92000 and 42000 obtained by incubation of the enzyme with proteinases *in vitro* (components C and E respectively of Fig. 1 in Nagler & Vartanyan, 1976; components i and ii of Fig. 1 of the present paper; see also Waud *et al.*, 1975; Mangino & Brunner, 1977a).

The lack of degradation products of xanthine oxidase in the skim-milk membrane fraction is of interest since this fraction is thought to derive from the sloughing of membrane from the apical surface of mammary secretory cells and from the surface of secreted fat globules (Plantz & Patton, 1973; Wooding, 1974; Kitchen, 1974; Wooding *et al.*, 1977). Presumably proteinases are not actively involved in these processes, otherwise xanthine oxidase would appear in a degraded form in this fraction. Skim milk also contains leucocytes with attendant lysosomal proteinases and the skim-milk-membrane fraction has been considered by some to be composed additionally of leucocyte membranes (Anderson & Cawston, 1975). This seems unlikely since leucocytic proteinases would be expected to degrade the membrane proteins, including xanthine oxidase. The membrane in skim milk therefore appears to be a source of membrane protein that is not as heterogeneous in origin or as degraded as originally suspected.

The monomeric molecular weight of xanthine oxidase in mammary tissue is the same as that in milk (Fig. 2). The enzyme is therefore secreted in an undegraded form and has a similar molecular weight to the xanthine dehydrogenases isolated from other

tissues [e.g., avian liver (Rajagopalan & Handler, 1967) and rat liver (Waud & Rajagopalan, 1976)]. Considering that at least half of the xanthine oxidase associated with fat-globules in milk is tightly bound to membrane, it is of great interest that approx. 80% of the enzyme in mammary tissue is in an apparently soluble form (see also Bailie & Morton, 1958). Since the specific activity of xanthine oxidase in fat-globule membrane is at least 70-fold higher than in microsomal membranes (Jarasch *et al.*, 1977) or 150-fold higher than in the postmicrosomal supernatant (I. H. Mather, C. H. Sullivan & P. J. Madara, unpublished work) the enzyme is evidently highly concentrated in a membrane-bound form during the secretion of milk-fat globules. This may be because the enzyme is a major component of the apical plasma membrane or that the enzyme, from either soluble or membrane-bound sources, accumulates around fat-globules within the cell before secretion. Further analysis of the location of xanthine oxidase in mammary tissue using immunocytochemical techniques may help to resolve these questions.

Note added in proof (Received 9 December 1981)

Since this manuscript was submitted for publication, a study on the localization of xanthine oxidase in mammary tissue using immunolocalization technique has been published (Jarasch *et al.*, 1981). Xanthine oxidase was detected throughout the cytoplasm of both endothelial and secretory cells, an observation in agreement with the soluble nature of the enzyme noted in the present study. In some secretory epithelial cells xanthine oxidase appeared concentrated in the apical regions, although the exact location of the enzyme with respect to intracellular fat globules remains unclear. An immunoblot of fat-globule membrane showed essentially one major immunoreactive band of estimated mol.wt. 155000 in agreement with Fig. 3(a) of the present paper.

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