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Sulfhydryl Oxidase in Human Milk: Stability of Milk Enzymes in the Gastrointestinal Tract

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Summary

Sulfhydryl oxidase (SOX) is present in human milk and in milk from all species that have been studied. The pH optimum of human milk SOX is in the neutral range between 7.0 and 7.5. Human milk SOX is stable in an acid environment: 50% of its activity remains after 1 h at pH 2.5. Acid stability is also characteristic of γ-glutamyltranspeptidase, another membrane-bound enzyme in skim milk. SOX is resistant to pepsin (4000 U/ml), trypsin (50 μg/ml), chymotrypsin (200 μg/ml), and to trypsin plus chymotrypsin (25 μg each/ml). Milk SOX activity has been detected in the stomach and proximal small intestine contents of suckling rats. Human and bovine SOX are relatively heat stable: 75% of the latter remains after treatment at 62.5°C for 30 min and 65% of the former remains after treatment at 60°C for 10 min. Neither remains after 62.5°C for 30 min.

Abbreviations

DTT, dithiothreitol
GGT, γ-glutamyltranspeptidase
GI, gastrointestinal
GSH, reduced glutathione
GSSG, oxidized glutathione
SIgA, secretory IgA
SOX, sulfhydryl oxidase

Some 60–70 enzymes have been reported to be present in milk (4, 35) but little is known about their function. Milk enzymes may act in the milk itself, in the intestine, or may be absorbed by intestinal epithelial cells and transported to target tissues. At least two human milk enzymes, bile salt-stimulated lipase and mammary amylase, have been shown to play a physiologic role in the digestion of milk nutrients (15, 18, 19, 22, 23, 28). It is unlikely, considering the strict control of secretory processes (29), that a significant number of enzymes occur in milk simply because of "spillage" from the blood or acinar cells.

Sulfhydryl oxidases are a class of enzymes that catalyze the *net* synthesis of disulfide bonds (21). These enzymes use molecular oxygen and both low molecular weight sulfhydryl compounds and protein sulfhydryls as substrates *in vitro* (3, 31, 34); they produce H_2O_2 plus a disulfide. Sulfhydryl oxidases were originally described in bovine milk (24) but have subsequently been found in skin (38), seminal vesicle (31), epididymis (6), and in mouse plasmacytomas producing IgM (34). The *in vivo* substrates for SOX in mouse plasmacytomas were shown to be IgM monomers and the associated J chain (34). But *in vivo* substrates for the sulfhydryl oxidases found in mammalian secretions and tissues have not been established. Milk SOX might play a role in altering the structure of milk or intestinal proteins that are dependent upon the redox state of their sulfhydryls for function.

The present experiments were designed to determine whether or not SOX and other milk enzymes had stability characteristics similar to those of milk proteins, such as secretory immunoglobulins (13, 25), which have been shown to be active in the gastrointestinal tract of the suckling neonate. Studies, both *in vitro* and *in vivo*, were done in order to measure the stability of SOX to acid and to gut protease.

MATERIALS AND METHODS

Human milk samples were obtained through the La Leche League of Staten Island. The milk was frozen immediately after collection from the donor. Informed consent was obtained from the donors by the La Leche League. Unprocessed bovine milk was obtained from a local dairy farm. Pregnant Sprague-Dawley rats and New Zealand white rabbits were obtained from Charles River Farms (Wilmington, MA). Pepsin, chymotrypsin, trypsin, dithiothreitol, glutathione, γ-glutamyl-*p*-nitroanilide, glycylglycine, *p*-dinitrophenylphosphate, NADH, and pyruvate were purchased from Sigma (St. Louis, MO).

Milk samples. Milk from rats and rabbits was obtained by anesthetizing lactating females by injection with 10 mg of sodium pentobarbital, stimulating milk secretion when necessary with oxytocin (0.5 IU in 0.5 ml of 0.9% saline) and then manually expressing the milk.

Enzyme assays. SOX activity was determined by measuring the disappearance of free substrate sulfhydryls at 37°C, using 5,5′-dithiobis (2-nitrobenzoic acid) (12). The enzyme assay was performed at pH 7.0 in 50 mM sodium phosphate buffer with DTT or GSH at a sulfhydryl concentration of 0.8 mM. A total reaction volume of 1.2 ml was used and 300 μL was taken for sulfhydryl determination. GGT (E.C. 2.3.2.2) was measured by the method of Griffith and Tate (16), using *p*-nitroanilide as substrate at 25°C. Alkaline phosphatase (E.C. 3.1.3.1) was determined at 25°C by the method of Lowry (27), which measures *p*-nitrophenol production. Lactate dehydrogenase (E.C. 1.1.1.27) activity was determined at 25°C by the disappearance of NADH absorption at 340 nm, using pyruvate as substrate (9). Proteins were measured by the method of Bradford (5).

Protease treatment of milk. For pepsin treatment, whole human milk was adjusted to pH 3.5 before pepsin was added. After a 2-h incubation at 37°C, pepsin digestion was stopped by raising the pH to 7.0 with 0.1 N NaOH. The milk was then spun at 16,000 *g* for 30 min at 4°C and the supernatant assayed for SOX. Trypsin and chymotrypsin were added to whole human milk and the milk was incubated at 37°C for 2 h. The milk was then cooled to 4°C spun at 16,000 *g* for 30 min and the supernatant was assayed for SOX. Protease concentrations used were in excess of those found in the GI tract of the newborn infant (1).

Heat treatment of milk. Milk samples were placed in a heat block set at the appropriate temperature and allowed to equilibrate; they were then kept at temperature for the indicated time.

Table 1. *Sulfhydryl oxidase (SOX) in milk**

Milk source	SOX activity: DTT utilization† [nmol/(min·mg protein)]	DTT utilization† [nmol/(min·ml milk)]	GSH utilization† [nmol/(min·mg protein)]	GSH utilization† [nmol/(min·ml milk)]
Human	39.3 ± 2.9 (7)	901 ± 66.0 (7)	0	0
Rat	94.5 ± 12.0 (3)	3273 ± 517 (3)	0	0
Rabbit	5.2	316	7.6	464
Bovine (dairy cow)	6.1 ± 1.2 (3)	204.6 ± 27.1 (3)	8.0 ± 1.8 (7)	234.9 ± 34.3 (7)
Bovine (colostrum)	4.5	148.6	7.5	250.4

* Values where indicated are mean ± SD. The numbers of samples assayed are indicated in brackets.

† Abbreviations: DTT, dithiothreitol and GSH, reduced glutathione.

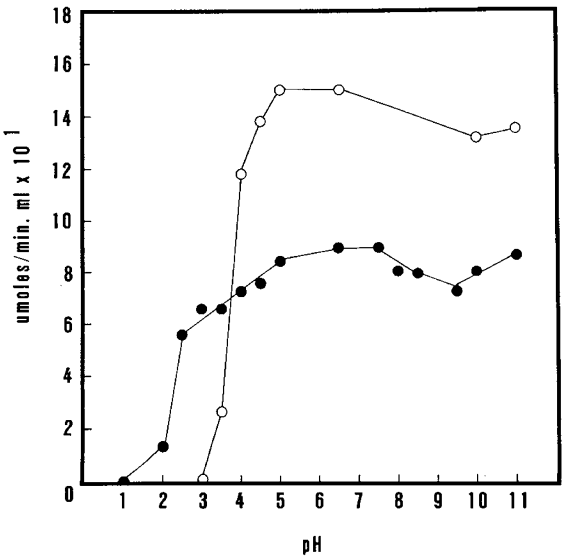

Fig. 1. pH stability of sulfhydryl oxidase (SOX) (●) and γ-glutamyl-transpeptidase (GGT) (○) in human milk.

pH stability studies. Milk was adjusted to the indicated pH with either 0.01 N HCl or 0.1 N NaOH and kept there for 1 h. It was then brought back to pH 7.0 and assayed for enzyme activity.

RESULTS

SOX is present in milk from all species that have been studied (7) (Table 1), including milk from lactating females without nursing offspring, *i.e.*, milk from dairy cows. Rat milk has the highest concentration of SOX, measured either as total SOX activity per ml or on the basis of protein concentration. SOX in human and rat milk utilizes reduced DTT as substrate but not GSH. Bovine and rabbit milk SOX, however, utilize both DTT and GSH as substrates and produce oxidized DTT and GSSG at almost equal rates. DTT-specific SOX has much greater activity, per ml milk or per mg protein, than SOX of the cow and rabbit milk. SOX both from human and from bovine milk is able to oxidize cysteine (data not shown). SOX activity is equivalent in bovine colostrum and dairy cow milk and has the same substrate specificity.

Human milk SOX activity is very stable in an acid environment (Fig. 1). No activity is lost at pH 4.0, 75–80% of the activity remains at pH 3.0, and greater than 50% resists exposure to pH 2.5. Human milk SOX is not inactivated at alkaline pH; greater than 90% of SOX activity remains at pH 11. Acid stability is also characteristic of GGT, another skim milk, membrane-bound enzyme (Fig. 1). GGT is not as acid stable as SOX but still retains greater than 70% of its activity at pH 4.0. The difference in acid stability between the two enzymes indicates that milk SOX activity does not require previous GGT activity, as has been suggested for renal SOX (16). At pH 3.5 and below, there is greater SOX than GGT activity on a molar basis.

Table 2. *Resistance of human milk sulfhydryl oxidase (SOX) to gastrointestinal protease*

Protease	SOX activity remaining [nmol/(min·ml)]
None	477.6
Pepsin	
(2000 U/ml)	486.8
(4000 U/ml)	205.9
Trypsin	
(50 μg/ml)	398.7
(200 μg/ml)	59.0
Chymotrypsin	
(50 μg/ml)	492.0
(200 μg/ml)	191.2
Trypsin + chymotrypsin	455.6
(25 μg each/ml)	93.8
(50 μg each/ml)	0
(100 μg each/ml)	

Studies were done in order to determine the susceptibility of SOX to GI proteases (Table 2). With 4000 U/ml of pepsin in human milk, 40–45% of SOX activity remains after 90 min. The enzyme is also relatively resistant to chymotrypsin. Trypsin at 50 μg/ml causes only minor loss of activity and even with 200 μg/ml of trypsin 10–15% of SOX activity remains. When trypsin and chymotrypsin were used together (25 μg/ml each), SOX was not inactivated; however, when 100 μg/ml of each was used for digestion all SOX activity was eliminated.

SOX from human and bovine milk is relatively heat stable (Table 3). Pasteurization of bovine milk (62.5°C, 30 min) inactivates only 25% of SOX activity. Human milk SOX is somewhat less heat stable than the bovine enzyme, but 65% of the activity remains at 65°C after 10 min. This degree of heat stability is also characteristic of some other human milk proteins that function in the GI tract, *e.g.*, lactoferrin and SIgA.

The pH optimum of human milk SOX is between 7.0 and 7.5 (Fig. 2). We were unable to find a second pH optimum below 6.0 (data not shown), which indicates that SOX would have almost maximal activity in the small intestine and partial activity within the bolus in the stomach.

Enzyme assays were performed on the stomach contents of 7- to 9-d-old suckling rats (Table 4). Activity was detected for the membrane-bound enzymes SOX, GGT, and alkaline phosphatase and for lactate dehydrogenase, the soluble milk enzyme. Measurements of the contents of the proximal small intestine indicated that rat milk SOX and GGT passed from the stomach into the intestine. Because SOX activity was measured with DTT, it is derived from milk and is not intestinal SOX activity, which utilizes GSH (16).

Table 3. *Heat treatment of human and bovine sulfhydryl oxidase (SOX)*

Treatment	% SOX remaining human milk	% SOX remaining bovine milk
None	100	100
50°C, 10 min	100	...
50°C, 20 min	59.5	...
60°C, 10 min	65	...
62.5°C, 30 min	0	75
65°C, 30 min	0	64
70°C, 30 min	0	18
100°C, 10 min	0	0

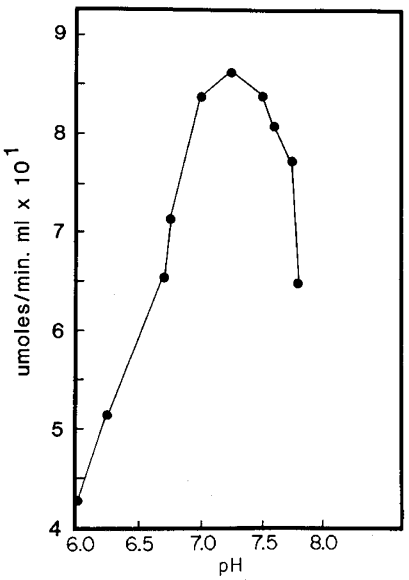

Fig. 2. pH optimum of human milk sulfhydryl oxidase (SOX). Assays were performed on milk that was centrifuged at 16,000 *g* for 30 min.

DISCUSSION

SOX has been found in the milk of all species tested (7) (Table 1), suggesting that this enzyme performs an important function in milk or in the suckling neonate of all mammals. The differences found in substrate specificity *in vitro* do not necessarily imply any substrate differences *in vivo*. All milk SOXs may perform the same *in vivo* function.

The acid stability of SOX and other milk enzymes and the resistance of SOX to heat denaturation are characteristics shared with other milk proteins that have been shown to be functional in the small intestine, *e.g.*, lactoferrin and SIgA (4, 25, 30). The pH optimum of SOX between 7.0 and 7.5 indicates that its activity in the gastric contents will be minimal because there is a pH range in the contents of 4.5–5.5 (17). It has been shown, though (Margit Hamosh, personal communication), that there is a pH gradient within the bolus in the stomach from 5.8–6.0 in the fundic area to 2.5 in the pyloric regions, suggesting that milk SOX may function in the bolus. But SOX could function optimally in the intestine, which has a neutral pH (10). The resistance of SOX to pepsin, trypsin, and chymotrypsin in our *in vitro* studies suggests that a significant quantity of the enzyme could remain intact in the intestine. Furthermore, all milks studied contain protease inhibitors, *e.g.*, anti-trypsin and anti-chymotrypsin (26), which may decrease the rate of proteolytic digestion in the intestine of the suckling neonate.

The presence of SOX, GGT, alkaline phosphatase, and lactate dehydrogenase in the stomach contents of suckling rats provides *in vivo* evidence that milk enzymes, in addition to lactoferrin and SIgA, are highly resistant to pepsin degradation. SOX and GGT activities in the contents of the proximal small intestine of suckling rats indicate that these two enzymes also are resistant to intestinal proteases and may function at some point in the intestinal tract. It is possible that GGT present in intestinal contents is derived from intestinal tissue; however, the SOX activity that we measured was DTT-specific, whereas "glutathione oxidase" (SOX?) found in small amounts in jejunal villus tip cells is specific for glutathione (39).

Table 4. *Milk enzymes in the gastroinestinal tract of 7- to 9-d-old rats**

Enzyme	Specific activity in stomach contents [nmol/(min·mg protein)]	Specific activity in proximal small intestine contents [nmol/(min·mg protein)]
SOX†	127.0 ± 24.8 (5)	154, 429
GGT†	207.0 ± 98.6 (3)	83, 77
Alkaline phosphatase	8.8 ± 2.5 (3)	...
Lactase dehydrogenase	191.2, 61.5	...

* Values where indicated are mean ± SD. Values in brackets are number of samples from different litters assayed.

† SOX, sulfhydryl oxidase and GGT, γ-glutamyltranspeptidase.

Milk SOX may have more than one function, as has been found with D-galactosyltransferase, another milk enzyme (36). The membrane-bound SOX induced in pentameric IgM-secreting mouse plasmacytomas, concomitantly with secretory heavy chain and J chain, catalyses the formation of disulfide bonds between IgM monomers and J chain. The primary immunoglobulin in human milk is SIgA, a dimer formed by disulfide linkage of IgA monomers to a J chain. Milk SOX may catalyse IgA dimer formation in the breast and then be secreted into milk with SIgA where it performs an additional function. Possible other roles for milk SOX are control of milk enzymes which are dependent upon the redox state of their sulfhydryls for function, *e.g.*, glucose-6-phosphate dehydrogenase (11), attachment of SIgA to intestinal mucins (40), and alteration of the mucous diffusion barrier that has been proposed by Smithson *et al.* (37). The protein component of mucins is a tetramer held together by disulfide linkages (2, 14, 32). Milk SOX has been shown *in vitro* to have the capability to oxidize not only low molecular weight thiols (3, 21) but also to reform correctly the disulfide bonds in chymotrysinogen A (20) and ribonuclease (21) and to convert milk xanthine oxidase from the dehydrogenase to the oxidase form by oxidation of two vicinal thiols (8). In the intestine, milk SOX may serve an interim role by supplementing immature intestinal or salivary enzyme. We have found SOX to be present in adult saliva [84 nmol/(min·ml)] but have not been able to detect SOX activity in saliva from 1- and 2-d old infants. SOX in milk may also have a function that is required solely in the immature small intestine.

High molecular weight proteins, such as IgG in suckling rats (33), can be absorbed by the proximal small intestine. The ability of suckling rats to absorb IgG decreases as the rat approaches weaning (21 d). It is thus possible that some milk enzymes may also be absorbed by the intestine and then transported by the circulatory system to their site of action. SOX may play a role in the intestinal absorption of proteins by altering the mucoprotein surface coat of the small intestine in suckling neonates. Experiments are presently underway to determine the site of action of milk SOX and to elucidate its *in vivo* substrate(s).

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