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Antibacterial Activity of the Purified Peroxidase from Human Parotid Saliva

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The peroxidase of human parotid saliva has been purified by concentration, gel filtration on Sephadex G-200, and ion exchange chromatography on Amberlite CG-50. The purified product was devoid of amylase activity, lysozyme activity, and immunoglobulin A (IgA). However, it had an inhibitory effect on the growth of *Lactobacillus acidophilus* in complete growth medium and on lysine accumulation by *L. acidophilus* in a buffer-glucose medium, when combined with thiocyanate ions. The concentrations of peroxidase and thiocyanate ions employed were within the range found in saliva. The fractions which contained IgA did not have an antibacterial effect on *L. acidophilus* under the conditions employed. Parotid saliva also contained low molecular weight inhibitors of peroxidase activity. These studies support the involvement of the salivary peroxidase in an antibacterial system in saliva.

There has been considerable interest in an antibacterial system in saliva which inhibits the growth of *Lactobacillus acidophilus* as well as a number of other organisms. This antilactobacillus system can be distinguished from lysozyme by heat sensitivity and organism susceptibility, and from formed elements in saliva such as bacteria or leukocytes by centrifugation or filtration (*see* 1). In 1959, Zeldow (21) reported that the antilactobacillus system of parotid or submaxillary saliva contained at least two components, a heat labile, nondialyzable component and a heat stable, dialyzable component. The heat stable, dialyzable component was reported by Dogon et al. (4) to be thiocyanate ions, and this was confirmed by Zeldow (22).

The nature of the heat labile, nondialyzable component was suggested by studies on a milk antibacterial system called lactenin. Hanssen (6) first suggested that the antibacterial activity of milk might be due, in part, to the "oxydases and peroxydases" of milk, since the antibacterial activity of milk in his test system and the "oxydases and peroxydases" had the same heat sensitivity. Wright and Tramer (20) confirmed this finding and found that the antibacterial activity and the peroxidase activity of milk also were affected similarly by variation of *p*H and by the addition of sodium azide and H_2O_2. Identification of lactoperoxidase as a component of the milk antibacterial system followed purification of the enzyme. Portmann and Auclair (14) demonstrated a direct relationship between the peroxidase activity of the fractions obtained in the purification process and their antibacterial activity. The antibacterial properties of a highly purified preparation of lactoperoxidase was confirmed by Stadhouders and Veringa (17) and by Jago and Morrison (8). The similarity between the milk and saliva antibacterial systems was emphasized by the finding by Reiter et al. (15, 16) that the milk antibacterial system required thiocyanate ions in addition to lactoperoxidase.

Klebanoff and Luebke (10) reported that lactoperoxidase, purified from bovine milk, could replace the heat labile nondialyzable component of the antilactobacillus system of saliva. Lactoperoxidase was employed because the peroxidase of saliva was not then available in purified form, and it had been reported that bovine salivary gland peroxidase and bovine lactoperoxidase were very similar if not identical enzymes (12). The purification of the peroxidase of human parotid saliva and the antibacterial properties of the purified product are described in this study.

MATERIALS AND METHODS

Collection of saliva. Parotid saliva was collected from normal human adults directly from Stensen's duct with the apparatus described by Curby (3). Salivary flow was stimulated with citric acid troches

(Sour Lemons, Regal Crown Co., England). The saliva was pooled and stored at 4 C until the desired volume was reached. All subsequent purification procedures were performed at 4 C.

Concentration. The parotid saliva was concentrated initially by ultrafiltration using a positive-pressure Diaflo Cell (model 400) containing a UM-2 filter (Amicon Corp., Cambridge, Mass.). The final stages of concentration were performed with a negative-pressure apparatus (membrane filter; Carl Schleicher & Schuell Co., Keene, N.H.) which combines ultrafiltration with dialysis. In this procedure, the collodion bag, which contains the solution to be concentrated, is immersed in an appropriate solution in the suction vessel. When collection of the ultrafiltrate is required, a collecting tube is fitted under the collodion bag in the suction vessel which, in this instance, does not contain fluid.

Chromatography. Gel filtration on Sephadex G-200 (Pharmacia, Inc., New Market, N.J.) was performed on a column (2.5 × 90 cm) equipped with an upward flow adaptor (Pharmacia) and a Beckman Accu-Flow pump. Next, 75-drop fractions (3.2 ml) were collected in a Beckman fraction collector (model 132). Ion exchange chromatography on Amberlite CG-50 (Mallinckrodt Chemical Works, St. Louis, Mo.) was performed on a 1.5 × 18 cm column. The resin was equilibrated at *p*H 7.0, and elution was performed in a stepwise fashion with increasing concentrations of sodium acetate (0.02 M, 0.25 M, and 0.5 M) as described for the purification of lactoperoxidase by Morrison and Hultquist (13). A higher sodium acetate concentration was initiated when no further 280 mμ absorbing material was eluted at the lower concentration. Fifty-drop fractions (2.1 ml) were collected.

Lysine accumulation. An overnight culture of *L. acidophilus* (ATCC #4357) in Lactobacillus Selective (LBS) broth (Baltimore Biological Laboratory) was washed two times with 0.02 M sodium acetate buffer (*p*H 5.0) containing 0.01 M glucose and was suspended in the same solution to an absorbancy of 0.250 in a Coleman Junior spectrophotometer (2×10^8 organisms per ml). The reaction mixture contained sodium acetate buffer (*p*H 5.0), 35 μmoles; *L. acidophilus*, 10^8 organisms; glucose, 7.5 μmoles; ^{14}C-lysine, 0.0005 μmole (0.1 μc); G-200 fraction, 0.01 ml; sodium thiocyanate, 1 μmole; H_2O_2, 0.1 μmole; and water to a final volume of 2.0 ml. Incubation was for 30 min at 37 C in an Eberbach water-bath shaker oscillating 120 times per min. The organisms were collected on a membrane filter and washed; the cell-associated radioactivity was determined as previously described (2).

Determination of bacterial growth. The inhibition of the growth of *L. acidophilus* in complete growth medium (LBS broth) was determined as previously described (10), except that a Coleman Junior spectrophotometer was employed. Viable cell counts were determined by the pour-plate method.

Agar gel diffusion. Human immunoglobulin G (IgG) was prepared by diethylaminoethyl (DEAE)-cellulose fractionation of human serum (5). The immunoglobulin A (IgA) fraction was isolated from human milk by starch block electrophoresis, ion-exchange chromatography on DEAE-cellulose, and Sephadex G-200 gel filtration (18). Antisera were prepared to the IgG and IgA in male New Zealand white rabbits. The antiserum to human IgA was absorbed with human cord serum. Rabbit antiserum to human immunoglobulin M (IgM) was obtained commercially from Behringwerhe (Hoechst Pharmaceutical Co., Cincinnati, Ohio). The three antisera were shown to react specifically with the corresponding antigens by immunoelectrophoresis and double diffusion in agar. The IgA, IgG, and IgM antisera were used to test the fractions of parotid saliva for the presence of immunoglobulin by double diffusion in agar.

Other procedures. Peroxidase activity was determined by the oxidation of *o*-dianisidine in the presence of H_2O_2 (9). One unit of activity is that causing an increase in absorbancy of 0.001 per min at 460 mμ in a Cary M-15 spectrophotometer. Protein was determined by the Lowry method (11). Lysozyme activity was determined by means of the lysozyme kit supplied by Worthington Biochemical Corp. (Freehold, N.J.) and amylase was determined by means of the amylase kit supplied by Sigma Chemical Co. (St. Louis, Mo.). Lactoperoxidase was prepared from bovine milk by the method of Morrison and Hultquist (13).

RESULTS

Table 1 demonstrates the results of a typical purification. The parotid saliva (250 ml, specific activity, 7.5 units per mg of protein) was concentrated 50-fold by a combination of positive pressure ultrafiltration and negative pressure ultrafiltration. The latter process combined ultrafiltration with dialysis against 0.1 M phosphate buffer, *p*H 7.0. A mucinous precipitate formed during the final stages of concentration. This precipitate was removed periodically by centrifugation of the concentrated saliva at 25,000 × *g* for 15 min. A considerable increase in the total peroxidase activity of the sample occurred during the concentration procedures (Table 1). The findings shown in Table 2 suggest that this increase in total activity was due, in large part, to the removal of low molecular weight inhibitors of peroxidase activity from saliva by dialysis. The peroxidase activity of parotid saliva, which generally ranged from 5 to 15 units per ml, was considerably increased by dialysis against water for 90 min. The peroxidase activity of the dialyzed preparation was inhibited by the addition of a

TABLE 1. *Purification of the peroxidase of human parotid saliva*

Fraction	Specific activity (units/mg of protein)	Total activity (units)	Purification
Parotid saliva	7.5	3,250	0
Concentration	148	12,250	20
G-200	943	8,052	126
CG-50	7,692	6,048	1,026

parotid saliva ultrafiltrate (Table 2). The removal of low molecular weight inhibitors by dialysis also would account, in part, for the apparent purification of peroxidase achieved by the concentration procedures (Table 1). Actual purification was achieved by the precipitation of protein during concentration and its removal by centrifugation.

The concentrated saliva (5 ml) was applied to a column of Sephadex G-200 and elution was performed with either 0.1 M phosphate buffer (*p*H 7.0) or 0.1 M acetate buffer (*p*H 5.0). Figure 1 demonstrates the absorbancy at 280 mμ, the peroxidase activity, and the antibacterial activity of the fractions. The absorbancy at 280 mμ and the peroxidase activity patterns were the same when elution was performed at either *p*H 7.0 or *p*H 5.0. The antibacterial activities were performed on fractions eluted at *p*H 5.0, since the *p*H corresponded to the *p*H of the assay.

Measurement of absorbancy at 280 mμ revealed the presence of three peaks. The first peak corresponded to the end of the void volume and thus represented substances excluded from the gel.

TABLE 2. *Peroxidase activity of saliva components*

Saliva components	Peroxidase activity (units/ml)
Whole saliva	5
Dialyzed saliva	62
Ultrafiltrate	0
Dialyzed saliva + ultrafiltrate	10

The bulk of the 280-mμ absorbing material was found in the third peak which contained amylase activity. The peak peroxidase activity was localized between the first and second protein peaks. The inhibitory effect of the fractions on lysine accumulation by *L. acidophilus* in the presence of thiocyanate ions and H_2O_2 was employed as a measure of antibacterial activity. The level of antibacterial activity corresponded closely to the peroxidase activity of the fractions.

On double diffusion with agar with concentrated parotid saliva, precipitin lines were obtained against the anti-IgG and anti-IgA sera but not against the anti-IgM sera. The fractions of the void volume peak and the peroxidase peak obtained from the Sephadex G-200 column were each pooled and concentrated 10-fold. The concentrated void volume peak formed a strong precipitin line against the IgA antisera but was devoid of peroxidase activity and did not inhibit lysine accumulation by *L. acidophilus* under the conditions employed. There was no precipitin line when the concentrated peroxidase peak was reacted against the IgA antisera. Neither concentrated peaks gave precipitin lines against IgG antisera.

Further purification of the salivary peroxidase was achieved by chromatography on the weak cation exchange resin, Amberlite CG-50. The pooled peroxidase fractions from the Sephadex G-200 column were dialyzed against distilled water for 4 hr and concentrated 20-fold with the negative pressure concentration apparatus which contained distilled water in the outer compart-

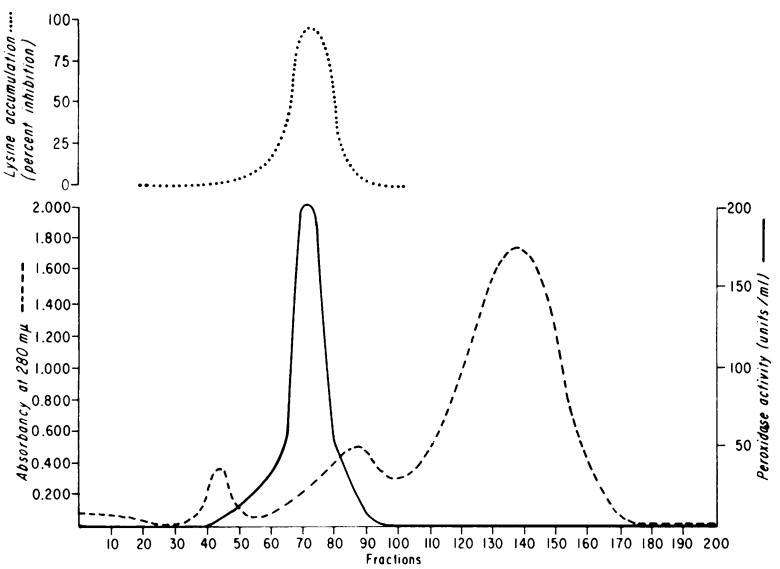

FIG. 1. *Gel filtration on Sephadex G-200.*

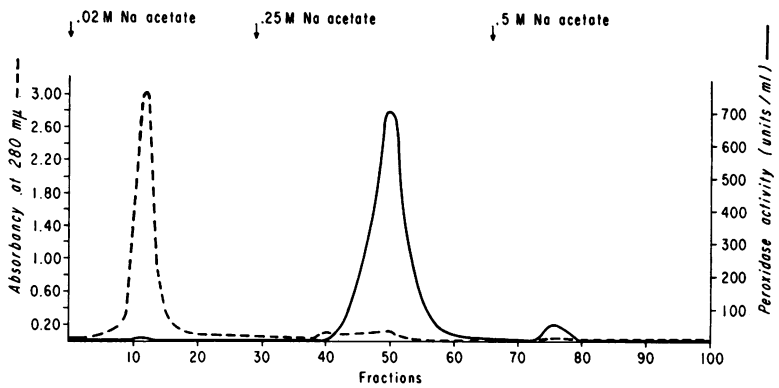

FIG. 2. *Ion exchange chromatography on Amberlite CG-50.*

ment. The concentrated preparation was applied to an Amberlite CG-50 column equilibrated at *p*H 7.0 and elution was performed with increasing concentrations of sodium acetate.

Elution with 0.02 M sodium acetate removed the bulk of the protein while the peroxidase was retained by the column (Fig. 2). The bulk of the salivary peroxidase was eluted with 0.25 M sodium acetate. A small peroxidase peak also was observed on subsequent elution with 0.5 M sodium acetate. The specific activity of the pooled large peroxidase peak was 7,692 units per mg of protein (Table 1).

The purified salivary peroxidase contained no detectable amylase activity, lysozyme activity, or IgA. The ratio of the absorbancy at 412 mμ to the absorbancy at 280 mμ of a concentrated preparation of the purified peroxidase was 0.68. This can be compared to the 412:280 ratio of 0.3 for the purified salivary antibacterial factor of Iwamoto et al. (7) and to the 412:280 ratio of 0.85 to 0.90 for the purified peroxidase of bovine milk (13) or of bovine submaxillary gland (12). Table 3 demonstrates the inhibitory effect of the purified salivary peroxidase and thiocyanate ions on the growth of *L. acidophilus* in complete growth medium (LBS broth; BBL). Both the peroxidase activity and the thiocyanate concentration were as found in parotid saliva. No inhibition of bacterial growth was observed when either the salivary peroxidase or thiocyanate ions were employed alone. However, when both were combined, the inhibition of bacterial growth was complete over the 24-hr period of measurement. The antibacterial activity of the salivary peroxidase was lost on heating at 75 C for 5 min. Lactoperoxidase, purified from bovine milk, could replace salivary peroxidase when added in amounts equal in peroxidase activity.

TABLE 3. *Inhibition of the growth of L. acidophilus by salivary peroxidase and thiocyanate ions*[a]

Supplements	Bacterial growth (absorbancy at 540 mμ)		
	0 hr	6 hr	24 hr
None	0.011	0.285	0.870
Thiocyanate	0.020	0.285	0.925
Salivary peroxidase	0.019	0.284	0.915
Salivary peroxidase + thiocyanate	0.026	0.025	0.026
Salivary peroxidase (heated) + thiocyanate	0.020	0.295	0.910
Lactoperoxidase	0.026	0.287	0.918
Lactoperoxidase + thiocyanate	0.027	0.026	0.026

[a] Reaction mixture contained 1.0 ml of double-strength LBS broth, 0.2 ml of a suspension of *L. acidophilus* (3.5×10^8 organisms per ml), water to a final volume of 2.0 ml, and supplements as follows: salivary peroxidase, 15 *o*-dianisidine units; lactoperoxidase, 15 *o*-dianisidine units; sodium thiocyanate, 1 μmole. The salivary peroxidase was heated at 75 C for 5 min where indicated.

DISCUSSION

The evidence suggestive of peroxidase involvement in an antibacterial system in saliva is substantial. It was previously reported that the heat-labile, nondialyzable component of the antilactobacillus system of saliva could be replaced by a purified preparation of the milk peroxidase, lactoperoxidase (10), an enzyme with very similar chemical and immunological properties to the salivary gland peroxidase (12). Indeed, the similarity of the peroxidases from the two sources has led to the designation of the salivary gland peroxidase as lactoperoxidase (12). Iwamoto et al. (7) reported that the salivary antibacterial factor purified from human whole saliva has peroxidase activity and spectral properties suggestive of a hemeprotein. We have reported here on the purification of the peroxidase of human parotid saliva and on the inhibitory effect of the purified product, when combined

with thiocyanate ions, on the growth of *L. acidophilus* in complete growth medium, and on the uptake of ^{14}C-lysine by organisms suspended in a buffer-glucose medium. The purified parotid saliva peroxidase and thiocyanate ions were employed in concentrations equivalent to those present in whole parotid saliva. The addition of H_2O_2 was not required when the test organism was *L. acidophilus*. *L. acidophilus*, however, are H_2O_2-generating organisms, and the inhibition of the antilactobacillus system of saliva by catalase (10) suggests that H_2O_2 of endogenous origin, presumably microbial, is required. The probable presence of low molecular weight inhibitors of peroxidase activity in saliva further emphasizes the complexity of the peroxidase-mediated antibacterial system.

It has been shown by other investigators that IgA is the predominant immunoglobulin of human parotid saliva (19). Our studies suggest that the peroxidase-mediated antibacterial system of saliva is independent of IgA. The purified peroxidase preparation did not contain detectable IgA, and the fractions of saliva which contained the highest concentrations of IgA did not have an antibacterial effect on *L. acidophilus* under the conditions employed. The purified peroxidase preparation also did not contain detectable IgG, was devoid of lysozyme activity, and did not contain any of the formed elements of saliva. Thus, the salivary peroxidase forms a component of an antibacterial system in saliva which is distinct from the other known salivary antibacterial systems.

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