Chromatographic separation of human salivary peroxidases

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Mäkinen, K. K. & Tenovuo, J. Chromatographic separation of human salivary peroxidases. *Acta Odont. Scand.* 34, 141—150, 1976.

A series of rapid and simple chromatographic purification procedures for peroxidase-like enzymes occurring in the human oral cavity is presented. Samples of whole saliva, parotid saliva, gingival exudate and various bacterial preparations contain peroxidases which were purified using molecular exclusion and ion exchange chromatography, and isoelectric focusing. Salivary lactoperoxidase can be easily separated from bacterial and leucocyte peroxidase activity by the methods presented.

Key-words: Peroxidases; saliva; dental plaque; gingival exudate

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Peroxidase activity in human whole saliva has been shown to comprise at least salivary lactoperoxidase, leucocyte myeloperoxidase, bacterial peroxidases and pseudoperoxidase activity (*Nickerson & Kraus*, 1955; *Nickerson, Kraus & Perry*, 1957; *Hosoya & Morrison*, 1965; *Morrison & Steele*, 1968; *Hoogendoorn*, 1974; *Mäkinen, Tenovuo & Scheinin*, 1975). At least lactoperoxidase may occur in different molecular forms (*Iwamoto et al.*, 1968; *Iwamoto et al.*, 1972). All three main pairs of salivary glands have been shown to secrete lactoperoxidase (*Nickerson & Kraus*, 1955; *Nickerson, Kraus & Perry*, 1957; *Morrison et al.*, 1965). Salivary lactoperoxidase is considered to play an important role in the antibacterial properties of the oral cavity. In spite of several studies the following shortcomings with regard to the knowledge of peroxidases of the human oral cavity still exist: 1. The main interest has so far been focused on the glandular peroxidases, i.e. the study of other sources, particularly microbial deposits, has partly been neglected. There is one additional source of oral peroxidases which was not included in the above papers, i.e. the gingival crevice fluid. 2. There has been no simple chromatographic method which would effectively differentiate between oral peroxidases of different sources. The aim of this paper is to describe simple chromatographic methods which can be used to differentiate the main sources of overall peroxidase activity in human oral fluid and certain other biological materials. The peroxidases revealed were identified on the basis of information and characteristics reported in the literature.

MATERIALS AND METHODS

1. Saliva and other samples

The following biological materials were studied: a) Supernatant fluid of whole

Received for publication, December 12, 1975.

mouth saliva; b) Parotid saliva; c) Gingival crevice fluid; d) Supernatant fluid of sonicated bacterial deposits (dental plaque); e) Supernatant fluid of plaque aqueous extract; f) Supernatant fluid of sonicated whole saliva sediment (representing largely a certain type of loose bacterial deposits, epithelial cells, leucocytes, precipitated mucins, etc.); g) Serum. The first six (a—f) were subjected to detailed chromatographic experiments (serum does not usually display sufficiently high peroxidase activity).

Most details of the sampling and treatment of the above materials for chromatography have been described earlier (*Mäkinen, Tenovuo & Scheinin*, 1975; *Mäkinen & Scheinin*, 1975 a; *Paunio, Mäkinen & Scheinin*, 1975; *Mäkinen & Scheinin*, 1975 b). Deviations are indicated in the Results section in the framework of the purification procedures. The form of the NaCl gradients was determined by atomic absorption spectrophotometry (Perkin Elmer Atomic Absorption Spectrophotometer Model 303).

2. Enzyme and protein assays

Peroxidase activity was determined by the guaiacol method as suggested by *Chance & Maehly* (1964). Pertinent information was also provided in other studies (*Mäkinen, Tenovuo & Scheinin*, 1975). Proteins were usually determined by the Lowry method (*Lowry et al.*, 1951). In most chromatograms the proteins are given in extinctions. In isoelectric focusing the proteins are given as transmittance at 278 nm. The peroxidases revealed were identified on the basis of their known molecular weights and chemical properties (*Mäkinen, Tenovuo & Scheinin*, 1975 a; *Mäkinen, Tenovuo & Scheinin*, 1975 b).

3. Chemicals

All reagents were of analytical grade or of the highest available purity. Guaiacol was a product of BDH Chemicals, Ltd. (Poole, England). $\beta\beta$-Dimethylglutarate was obtained from Calbiochem (Luzern, Switzerland). DEAE- and CM-cellulose were obtained from Schleicher & Schüll (Kassel, Einbeck, Germany). Unless otherwise mentioned, other chemicals were products of E. Merck AG (Darmstadt, Germany). The water used in the study was distilled and passed through ion exchange columns (specific resistance approximately 1 MΩ-cm).

RESULTS

Purification procedures

1. Whole saliva

1) *Collection and pretreatment.* 2300 ml of paraffin-stimulated whole saliva (55 persons, equally of both sexes) was centrifuged for 20 min at 18000 rpm (46000 $\times$ g) in cold (+ 4°C). The pellets were collected for the purification of the sediment peroxidases. The saliva can also be collected in smaller portions and stored at —20°C for several months until a sufficient amount is obtained. No noticeable loss of activity is usually observed.

2) *Concentration.* The supernatant fluid from the above step was freeze-dried to dryness or to 80—90 ml. The solid residue after freeze-drying was dissolved in 35 ml of cold (+ 4°C) water. The resulting mixture (in case of turbidity) was centrifuged for 30 min at 46000 $\times$ g. If the supernatant fluid is concentrated to 80—90 ml, it can be directly fractionated as indicated below (after the removal of any turbidity).

3) *Molecular permeation chromatography.* The supernatant fluid of the previous step is usually too viscous for this purpose.

Fig. 1. Molecular exclusion chromatography of peroxidases of concentrated supernatant fluid in human whole mouth saliva, determined according to the guaiacol method.

A: Chromatography on Sephadex G-100 Superfine. Column: 4.2 × 94 cm; Elution buffer: 0.01 M $\beta\beta$-dimethylglutarate buffer, pH 7.2; Sample: 25 ml of concentrated supernatant fluid of saliva mixed with 2 ml of 0.5 % Blue Dextran solution; Hydrostatic pressure: 15 cm; Temperature: +4°C; Flow rate: 0.7 ml per min; Fraction volume: 2.5 ml. Fractions 10—130 were pooled. Enzyme activity is given in seconds needed to reach an absorbance of 0.050 in the reaction mixture after the addition of hydrogen peroxide (*Mäkinen, Tenovuo & Scheinin,* 1975 a). Proteins are given in extinctions (Folin Ciocalteu reagent).

B: Chromatography on Sephadex G-200. Column: 4.2 × 92 cm; Sample: 22 ml (see text); Flow rate: 0.2 ml per min; other details as for A. Fractions 25—165 were pooled.

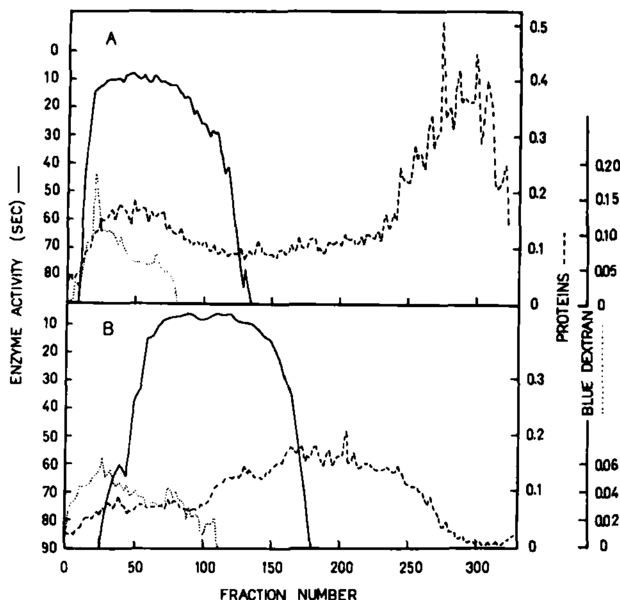
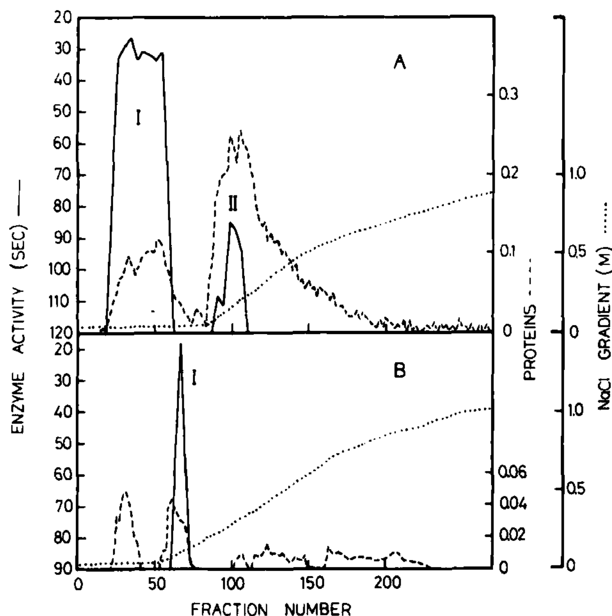


Fig. 2. A. DEAE cellulose chromatography of the pooled fractions of molecular exclusion chromatography (see text and Fig. 1). Column: 2.0 × 36 cm; Elution buffer: 0.01 M $\beta\beta$-dimethylglutarate buffer, pH 7.2, containing a linear NaCl gradient from 0 M to 1.0 M (mixing volume 300 + 300 ml); Flow rate: 0.2 ml per min; Hydrostatic pressure in packing and elution: 120 cm; Fraction volume: 3.0 ml; Temperature: +4°C. The sample (27 ml) was described in text. The sample was desalted with Sephadex G-25 gel (column: 5.5 × 32 cm). Fractions 20—63 (Peak I) and 87—110 (Peak II) were pooled (Fig. 2 B).

B. CM cellulose chromatography of the pooled fractions of DEAE cellulose chromatography (see text and Fig. 2 A). Column: 1.8 × 38 cm; Flow rate: 1.0 ml per min; Mixing volume of the NaCl gradient 250 + 250 ml; Fraction volume: 2.5 ml; other details as for A.



In the present method the enzyme preparation was diluted with cold water to 80—90 ml (or freeze-drying was interrupted earlier; the above centrifugation is still necessary). This solution was applied on a Sephadex G-100 Superfine column (chromatography on Sephadex G-200 may result in partial overlapping of most proteins and the peroxidase activity if a column of the type mentioned in Fig. 1 is used). Typical chromatograms are shown in Fig. 1.

4) *Concentration.* The active fractions (10—130 in the present study) of the molecular permeation chromatography were pooled, the pool was freeze-dried, and the solid residue was dissolved in 27 ml of cold 0.01 M $\beta\beta$-dimethylglutarate buffer, pH 7.2 (or corresponding phosphate buffer). The resulting mixture was centrifuged for 20 min at $46000 \times g$. The supernatant fluid was used in the next step.

5) *DEAE-cellulose chromatography.* The preparation of the previous step was treated with a Sephadex G-25 column (see legend to Fig. 2). The desalted preparation was applied on a DEAE-cellulose column (Fig. 2 A). The conditions involved usually led to the appearance of two peroxidase peaks (I and II). The conditions presuppose the use of Schleicher & Schüll cellulose, 230—270 mesh. A fine grade (over 270 mesh) evidently results in better resolution and higher heterogeneity. Peak I contained true salivary lactoperoxidase. This enzyme was further purified as follows.

6) *CM-cellulose chromatography* The active fractions of the previous step were pooled and the solution was freeze-dried. The solid residue was dissolved in 7 ml of the above buffer and the mixture was centrifuged (20 min at $46000 \times g$). The supernatant fluid was applied on a CM-cellulose column (Fig. 2 B). The resulting salivary lactoperoxidase preparation was suitable for many enzyme studies. The development of the specific activity is indicated in Table I. If higher purity is required, the preparation can be focused (see below).

Table I. *Specific activity (in units permgprotein) of certain peroxidase preparations obtained in the purification of whole saliva peroxidases*

Preparation	Activity
(1) Original supernatant fluid of whole saliva	11.1
(2) Concentrated preparation (freeze-drying of (1)	39.0
(3) Pool I after Sephadex G-100 Superfine (Fig. 1 A)	28.4
(4) Concentrated preparation of (3) (freeze-drying)	16.3
(5) DEAE-pool I (Fig. 2 A)	42.2
(6) DEAE-pool II (Fig. 2 A)	2.5
(7) Concentrated DEAE Pool I (freeze-drying)	83.8

It was found that Peak II in Fig. 2 A (DEAE-cellulose chromatography) contains two peroxidases, which are not separated in the subsequent CM-cellulose chromatography. When the enzyme peaks of Fig. 2 A were focused in a pH gradient from 3.5 to 10, both produced a lactoperoxidase peak with the same isoelectric point of 8.1 (Fig. 3). The enzyme with an IP of 4.3 most likely represented a mixture of bacterial, pseudoperoxidase and other peroxidase activities. It may also have contained heterogenic forms of lactoperoxidase.

2. Parotid saliva

1) *Collection and pretreatment.* 250 ml of parotid saliva (5 persons, 3 males, 2 females) was concentrated with an Amicon Ultrafiltration System TCF-10 (Membrane UM20E) to 25 ml and the concentrate was

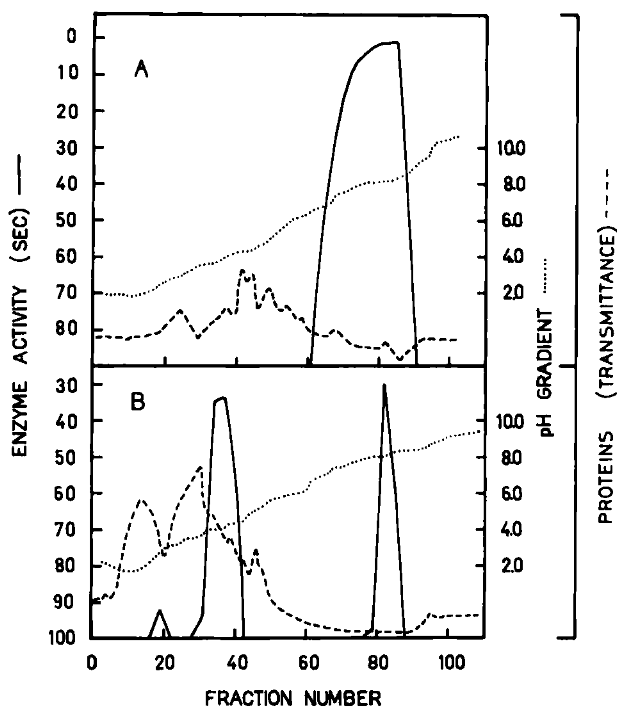


Fig. 3. Isoelectric focusings of concentrated and dialyzed pools from DEAE cellulose chromatography (see text). pH range from 3.5 to 10. Fraction volume: 1.1 ml; Voltage: 350 V; Current, <10 mA. A: Pool I of Fig. 2 A. B: Pool II of Fig. 2 A.

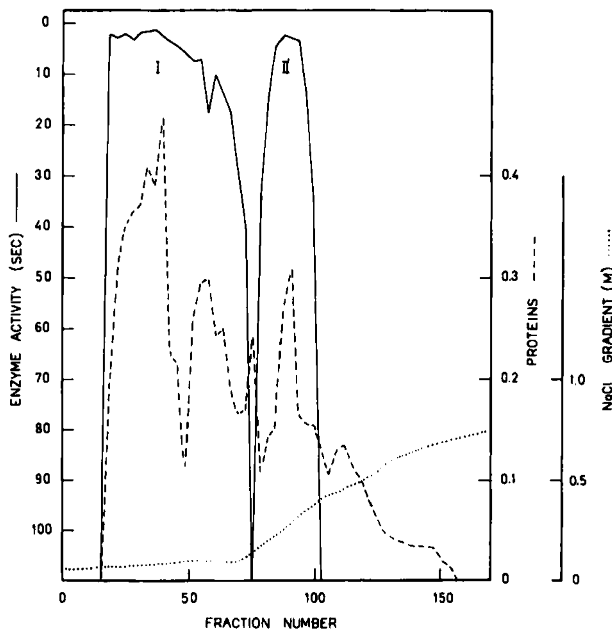


Fig. 4. DEAE cellulose chromatography of dialyzed human parotid saliva. Column: 1.7 × 18 cm; Mixing volume of NaCl gradient: 150 + 150 ml; Fraction volume: 1.5 ml; Sample: 40 ml of concentrated and dialyzed human parotid saliva (see text). Other details as for Fig. 2. Fractions 16—74 (Peak I) and 76—102 (Peak II) were pooled.

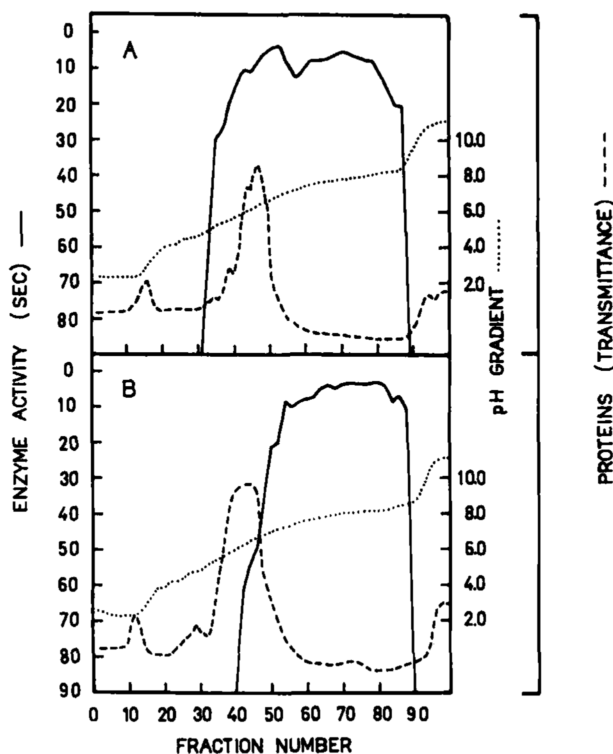


Fig. 5. Isoelectric focusings of concentrated and dialyzed pools from DEAE cellulose chromatography (parotid saliva; Fig. 4). All details as for Fig. 3. A: Peak I of Fig. 4. B: Peak II of Fig. 4.

subsequently dialyzed in the same device against 500 ml water.

2) *DEAE-cellulose chromatography.* The resulting solution was applied on a DEAE-cellulose column (Fig. 4). The active fractions forming the peaks (I and II) were separately pooled. The pools were concentrated as above and the resulting preparations were focused (Fig. 5).

As with whole saliva, Peak I of the parotid fluid yielded salivary lactoperoxidase with an isoelectric point of 8.1. Peak II of Fig. 4 yielded in isoelectric focusing in this study a broad peak containing two partially overlapping enzymes with isoelectric points of 7.0 and 7.8. The above two subsequent chromatographic steps (DEAE-cellulose and isoelectric focusing) produced a salivary lactoperoxidase with satisfactory purity. The single peak of Fig. 5 A, contained, however, at least two enzymes (lactoperoxidases). Their separation was performed as follows:

3) *Molecular permeation chromatography.* The active fractions of the isoelectric focusings (Fig. 5 A) were pooled, and the ampholytes and sucrose were removed with a Sephadex G-50 column (4.5 cm × 32 cm). The active fractions from this step were also pooled and freeze-dried. The solid residue was dissolved in 2.0 ml buffer and the resulting preparation was applied on a Sephadex G-100 Superfine column (Fig. 6). Peak I (lactoperoxidase) produced two active forms, one almost coinciding with the void volume and one with a molecular weight of 78000. The latter peak was considered to represent a monometric form of the enzyme. Peak II of Fig. 5 B produced only one active peak, which coincided with the void volume (Fig. 6 B).

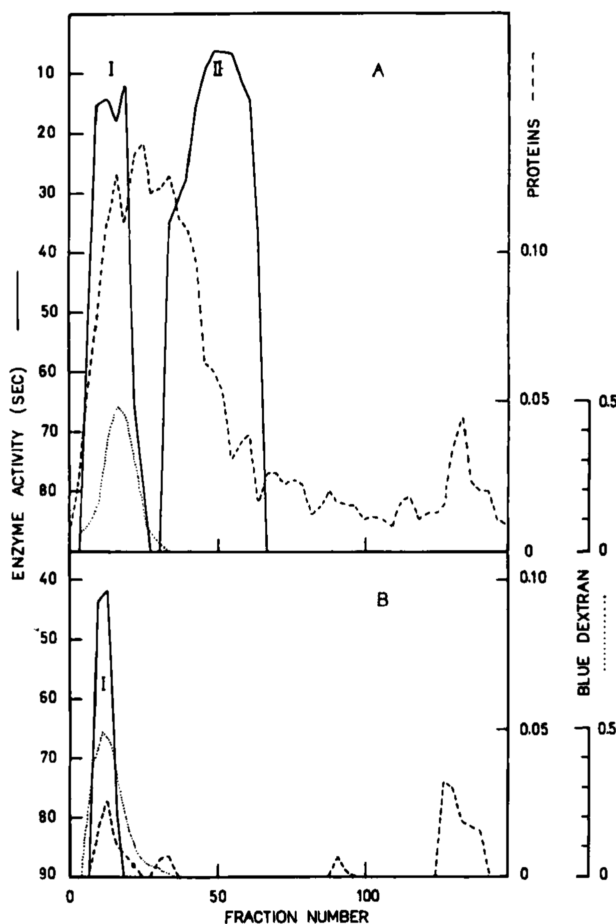


Fig. 6. Molecular exclusion chromatography of the pools obtained from isoelectric focusing (Fig. 5).
A: Column: 1.8 × 132 cm; Elution buffer: 0.01 M ββ-dimethylglutarate buffer, pH 7.2; Sample: 2.0 ml of the focusing pool (Fig. 5 A); Fraction volume: 0.7 ml; Hydrostatic pressure: 15 cm; Temperature: +4°C; Flow rate: 0.03 ml per min. Fractions 4—25 (Peak I) and 31—67 (Peak II) were pooled.
B: Column: 1.8 × 128 cm; Sample: 2.0 ml of the focusing pool (Fig. 5 B). All other details as for A. Fractions 7—18 were pooled.

Consequently, in this study both peroxidase peaks of Fig. 4 yielded one peroxidase with a high molecular weight after focusing. Table II shows the development of the specific activities.

3. *Gingival crevice fluid*

The single peroxidase usually present in detectable amounts in human gingival crevice fluid (gingival exudate) was purified as follows:

1) *Collection and pretreatment.* Samples of 32 persons (equally of both sexes), obtained by the filter paper method (*Paunio, Mäkinen & Scheinin,* 1973) were extracted for 5 min from the paper strips

Table II. *Specific activity (in units per mg protein) of certain peroxidase preparations obtained in the purification of parotid saliva peroxidases*

Preparation	Activity
(1) Original parotid saliva	15.1
(2) Concentrated sample	30.8
(3) DEAE-Pool I (Fig. 4)	10.0
(4) Focusing Pool of (3) (Fig. 5 A)	64.8
(5) Sephadex G-100 Superfine Pool I of (4) (Fig. 6 A)	5.80
(6) Sephadex G-100 Superfine Pool II of (4) (Fig. 6 A)	10.5
(7) DEAE-Pool II (Fig. 4)	18.3
(8) Focusing pool of the (7) (Fig. 5 B)	1.5
(9) Sephadex G-100 Superfine Pool I of preparation (8) (Fig. 6 B)	33.0

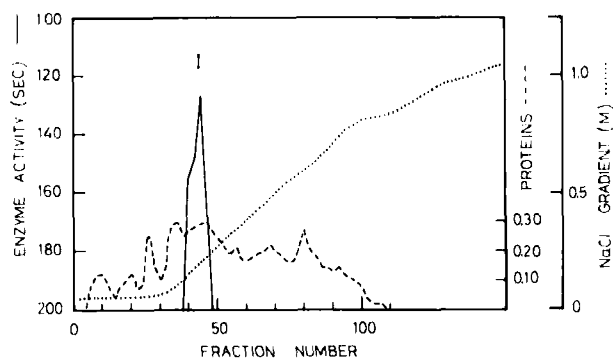


Fig. 7. DEAE cellulose chromatography of gingival exudate peroxidases. Sample: 14 ml of concentrated and dialyzed gingival crevice fluid (see text). All details as for Fig. 4.

in 25 ml of cold buffer. The mixture was centrifuged for 20 min at 46000 $\times g$. The supernatant fluid (I) was collected (specific activity 3.46 units per mg). The pellet was suspended in 4.0 ml of the above cold buffer, the mixture was centrifuged as above, and the supernatant fluid (II) was collected. I and II were pooled. After concentration (to 10 ml) and dialysis with Amicon ultrafiltration system (against 200 ml water), the preparation was applied on DEAE-cellulose column (Fig. 7). The specific activity of this preparation in this study was very low (approximately 3.5 units per mg protein). The peroxidase activity obtained from gingival exudate most likely represented the leucocyte myeloperoxidase (*Nickerson & Kraus*, 1955; *Mäkinen, Tenovuo & Scheinin*, 1975 a).

4. Bacterial deposits (dental plaque)

The human oral micro-organisms evidently contain a number of various proteins displaying peroxidase activity. The plaque mass, collected *in situ* (*Scheinin & Mäkinen*, 1971), was divided into two fractions: the aqueous extract and the cellular fraction. The cellular fraction contains a peroxidase which was purified as follows.

1) *Collection and pretreatment.* 0.3 g of dental plaque (wet weight), representing approximately 30 persons, was suspended in 5.0 ml buffer. The mixture was stirred with a glass rod for 5 min and centrifuged for 20 min at 46000 $\times g$. The supernatant fluid (specific activity approximately 5.0 units per mg) was used to determine the peroxidase activity of the aqueous extract. Subsequent DEAE-cellulose chromatography was used for the separation of a peroxidase which was most likely of salivary origin.

2) *DEAE-cellulose chromatography.* The pellet remaining after the above centrifugation (step 1) represented the plaque cellular fraction. The pellet was suspended in 5.0 ml of 0.01 M $\beta\beta$-dimethylglutarate buffer, pH 7.2. The mixture was sonicated for 10 min with an MSE Ultrasonic Disintegrator (100 W Modell, end diameter of the probe 9 mm). The mixture was kept in ice during the treatment. The resulting preparation was centrifuged, treated with a Sephadex G-25 (Coarse) column (5.5 $\times$ 30 cm; elution with the aid of the above buffer). The desalted crude enzyme solution (50 ml) was concentrated. Chromatography on DEAE-cellulose resulted in the separation of microbial peroxidases (Fig. 8). The salivary sediment can also be treated in the above way to yield microbial peroxidases. DEAE-cellulose chromatography of the salivary

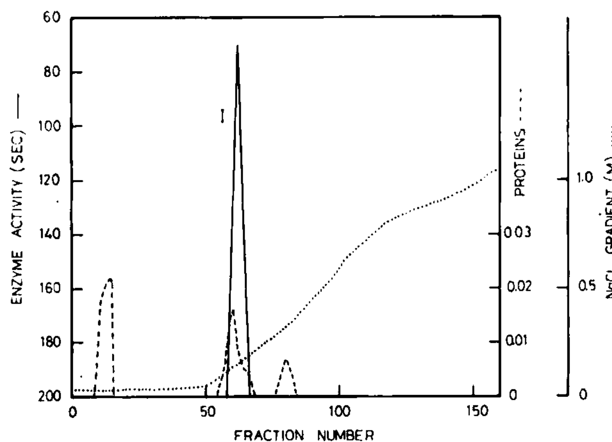


Fig. 8. DEAE cellulose chromatography of sonicated bacterial deposits (dental plaque). Sample: 37 ml of the supernatant fluid of sonicated dental plaque (see text). All details as for Fig. 4.

sediment peroxidases produced the same kind of chromatographic pattern as that of dental plaque.

5. Serum

Serum peroxidase activity was very low. No purification of peroxidase was possible.

DISCUSSION

Salivary lactoperoxidase and bacterial and leucocyte peroxidases differ from each other in their fractionation qualities, facilitating their simple and rapid purification. Freeze-drying and ultrafiltration were considered as alternative methods in the present study. When used in the way described, no noticeable differences could be observed between these methods with regard to the preservation of the enzyme activity.

The salivary sediment may be considered to represent a microbial preparation of the oral cavity in the same way as dental plaque, although the former also contains a low number of ingredients of salivary origin. This may occasionally result in a low lactoperoxidase activity in chromatograms obtained from the salivary sediment. The purification procedures described clearly indicated, however, that the salivary peroxidase was practically totally missing in the salivary sediment material, which contains bacteria and leucocytes. Nor was this enzyme present in serum, bacterial deposits or gingival exudates. Lactoperoxidase was clearly present in higher amounts only in fractionations of the supernatant fluid of whole saliva. The present methods can be used in large scale purification as well.

The heterogeneity of parotid saliva lactoperoxidase, found in this study, has also been observed earlier (*Iwamoto et al.*, 1968; *Iwamoto et al.*, 1972). It is likely that the present methods do not reveal all peroxidases of the bacterial deposits, because most bacteria do not react to guaiacol (*Soininen & Ellfolk*, 1972). The cells of a cariogenic strain of *Streptococcus mutans* investigated in this laboratory behave in this way.

Acknowledgements. The authors are indebted to Prof. K. U. Paunio for the collection of the gingival crevice fluid samples and to the Finnish Dental Society for a grant to the second author.

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