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Purification and Characterization of Human Salivary Peroxidase†

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ABSTRACT: Human salivary peroxidase (SPO) has been purified to homogeneity by subjecting human parotid saliva to immunoaffinity, cation exchange, and affinity chromatography. These procedures resulted in a 992-fold purification of the enzyme. When purified SPO was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), three Coomassie stainable bands were apparent, all of which stained positive for enzyme activity. The apparent molecular weights of the three bands were 78 000, 80 000, and 280 000 as analyzed by SDS–PAGE. Reduction with 2-mercaptoethanol resulted in a decreased mobility of these bands, and enzyme activity could no longer be detected on the gels. The SPO preparation had the characteristic peroxidase heme spectrum in the range 405–420 nm. The ratio between the absorbance of the Soret band (412 nm) and the absorbance at 280 nm was 0.81. The enzyme activity was inhibited by the classical peroxidase inhibitors cyanide and azide. Salivary peroxidase is similar to bovine lactoperoxidase (LPO) in amino acid composition, in ultraviolet and visible spectrum, in reaction with cyanide, in susceptibility to 2-mercaptoethanol inactivation, and in thermal stability. The two enzymes differ in carbohydrate composition and content. SPO contains 4.6% and LPO 7% total neutral sugars. The ratio of glucosamine to galactosamine is 2:1 in SPO and 3:1 in LPO. SPO contains mannose, fucose, and galactose in a molar ratio of 1.5:1.5:1.0, while the ratio was 14.9:0.5:1.0 in LPO. Glucose was present in both preparations in minor amounts. The concentration of azide required for 50% inhibition of enzyme activity was 20-fold greater for LPO than for SPO. The specific activity of SPO is approximately 5 times higher than that of LPO when 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) is used as a substrate.

The peroxidase in human saliva is one of the nonimmunoglobulin defense factors found in mucosal secretions [for review, see Tenovuo and Pruitt (1984) and Mandel and Ellison (1985)]. The enzyme catalyzes the oxidation of the thiocyanate ion by hydrogen peroxide to generate oxidized forms of the thiocyanate ion (Thomas, 1985). Several studies have suggested that the peroxidation reaction serves at least two important functions in the human mouth: (i) The products of the reaction inhibit bacterial growth and metabolism [for review, see Pruitt and Reiter (1985)], and (ii) the reaction prevents the accumulation of hydrogen peroxide excreted by many strains of oral streptococci (Thomas et al., 1983; Carlsson et al., 1983) and by host cells (Pruitt et al., 1983). Hydrogen peroxide is highly toxic for mammalian cells, but it is consumed rapidly by the peroxidation reaction, the products of which are nontoxic (Hanstrom et al., 1983; White et al., 1983; Tenovuo & Larjava, 1984).

Both salivary peroxidase (SPO) and lactoperoxidase (LPO) have catalytic properties that are qualitatively similar to those of other mammalian peroxidases, e.g., human myeloperoxidase (Clark et al., 1975), cervical mucus peroxidase (Shindler et al., 1976), and eosinophil peroxidase (Wever et al., 1980). All of these enzymes catalyze the oxidation of halides and thiocyanate in the presence of hydrogen peroxide. However, myeloperoxidase and eosinophil peroxidase catalyze the oxidation of chloride, but LPO and SPO do not.

Although attempts have been made to isolate the peroxidase from human saliva, only partially purified preparations have been obtained, and only limited biochemical characterization has been reported (Morrison & Allen, 1963; Iwamoto & Matsumura, 1966; Slowey et al., 1968). The difficulty of purification is partially due to the fact that the enzyme, at a concentration of 1–10 μg/mL (Tenovuo, 1985), is a relatively minor component of saliva [total protein concentration = 2.8–3.2 mg/mL (Nikiforuk, 1985)]. Furthermore, there is probably low motivation for undertaking the isolation of SPO because of similarities in catalytic properties of SPO and the more readily available bovine LPO (Morrison & Steele, 1968).

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The latter has been thoroughly studied by many investigators (Rombauts et al., 1967; Carlstrom, 1969; Sievers, 1981; Wever et al., 1982; Bardsley, 1985).

Our previous studies of partially purified human SPO (Mansson-Rahemtulla et al., 1985) indicated that this enzyme differs from bovine LPO in chemical, immunobiological, and kinetic properties. A major long-term goal of our work is to relate the structure of secretory peroxidases to their function. In the present paper, we describe the isolation, purification, and chemical characteristics of human salivary peroxidase. A detailed analysis of the kinetic properties of both SPO and LPO will be described in an accompanying paper (Pruitt et al., 1988).

Experimental Procedures

Purification Procedure. Stimulated parotid saliva was collected from healthy donors by use of Lashley cups (Lashley, 1916) as previously described (Pruitt et al., 1983). Approximately 100 mL of saliva was collected on each day of donation. Immediately after collection, samples were analyzed for peroxidase activity, absorbances were measured at 280 nm, and protein concentrations were determined with bovine serum albumin as a reference standard. Approximately 1 L of saliva was collected from each donor over a 5-week period. The saliva was lyophilized and stored at −20 °C. Samples from three different donors were stored and subsequently processed separately. All subsequent purification steps were carried out at 4 °C unless noted otherwise.

The lyophilized saliva, 12.67 g (obtained from 1 L), was dissolved in 200 mL of PBS[1] (0.01 M potassium phosphate, 0.15 M sodium chloride) containing 0.05 M EDTA, pH 5.4 (PBS–EDTA), and centrifuged at 15000*g* at 4 °C for 30 min. The resulting pellet contained less than 1% of the total enzyme activity and was discarded. The supernatant was dialyzed extensively against PBS–EDTA, assayed for peroxidase activity and protein content, and divided into four portions, which were stored at −20 °C.

Polyclonal antibodies against bovine LPO (purity index: A_{412nm}/A_{280nm} = 0.92) were raised in rabbits and have been partially characterized (Mansson-Rahemtulla et al., 1985). The IgG fraction from the rabbit antiserum was isolated by ammonium sulfate precipitation (Garvey et al., 1983) and coupled to cyanogen bromide activated Sepharose 4B according to the manufacturer's instructions (*Affinity Chromatography; principles and methods*, Pharmacia Fine Chemicals, 1979). The coupled gel was stored in PBS–EDTA. Final concentration of IgG coupled to the gel was approximately 11 mg/mL of gel.

A fraction of the dialyzed sample, with an activity of approximately 200 mmol/min of enzyme activity as determined by the NbsSCN assay at pH 6.8 (see below), was applied to the IgG–Sepharose 4B column (5 × 5 cm), and the unbound proteins were removed by washing the column with 30 volumes of PBS–EDTA. The peroxidase was eluted with 3.0 M sodium thiocyanate in PBS, pH 7.0. The flow rate was 25 mL/h, and 8.3-mL fractions were collected. The enzyme-containing fractions were pooled, dialyzed extensively against quartz distilled water, and lyophilized.

The lyophilized crude enzyme preparation from the immunoaffinity chromatography step was dissolved in 0.02 M sodium acetate buffer, pH 7.0, and applied on a column (2.5 × 10 cm) of CM-Sepharose Fast Flow, which had been equilibrated with the same buffer. The column was washed with 500 mL of the acetate buffer at a flow rate of 75 mL/h, and 5.2-mL fractions were collected. The peroxidase was eluted from the column with 0.6 M sodium acetate, pH 7.0, and the enzyme-containing fractions were pooled and dialyzed extensively against 0.05 M Tris-HCl, pH 7.4, for the next chromatography step.

In a preliminary set of experiments Con A–Sepharose 4B, phenyl-Sepharose, and Blue Sepharose CL-6B were evaluated for the last purification step. Consequently, the peroxidase preparation from cation-exchange chromatography was applied to a column (2.5 × 15 cm) of Blue Sepharose CL-6B, which had been equilibrated in 0.05 M Tris-HCl, pH 7.4. The column was washed with 750 mL of the starting buffer at a flow rate of 30 mL/h, and 5.0-mL fractions were collected. The peroxidase was eluted with the Tris-HCl buffer containing 0.6 M NaCl. The enzyme preparation was rechromatographed on the Blue Sepharose column under conditions identical with those described above to increase the purity of the sample.

Enzyme Assays. In the NbsSCN assay system peroxidase activities were determined by measuring the rate of oxidation of thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$) (Aune & Thomas, 1977). The assay conditions have been described in detail (Mansson-Rahemtulla et al., 1986), but the following modifications have been employed in this study. The pH of the buffer was 6.8, and the final concentration of SCN^- was 89.5 mM.

The enzyme activity was expressed as micromoles of Nbs oxidized per minute using an extinction coefficient of 14.258 mM^{-1} cm^{-1} for NbsH at pH 6.8 (Mansson-Rahemtulla et al., 1986). This pH and this SCN^- concentration were used for all samples except the fractions eluted from the immunoaffinity column. For these fractions, it was necessary to modify the assay conditions for the following reasons. The elution was carried out at [SCN^-] = 3.0 M. Dilution into the assay mixture of the fractions containing this concentration of SCN^- would give [SCN^-] = 89.5 mM. Since high SCN^- concentrations inhibit the enzyme activity (Pruitt et al., 1988), the measured activities of the fractions would vary with [SCN^-] as well as with enzyme activity. We avoided this complication by measuring the actual [SCN^-] in each fraction and adding sufficient SCN^- as required to give a final [SCN^-] of 89.5 mM in the assay mixture for all fractions analyzed from the immunoaffinity column.

Peroxidase activity was also measured with ABTS as a substrate. The assay conditions have been described by Shindler et al. (1976). One enzyme unit is equivalent to an increase in absorbance of 32.4/min at A_{412nm} and corresponds to the amount of enzyme catalyzing the oxidation of 1 mM of the ABTS–substrate under the described assay conditions. This definition assumes an absorption coefficient of 32400 M^{-1} cm^{-1} for ABTS at 412 nm (Mansson-Rahemtulla et al., 1986).

The data for both the enzyme assays were obtained from a Cary Model 219 spectrophotometer (Varian) interfaced with an PDP-11/73 computer system. Enzyme activities were calculated from slopes determined by least-squares analysis of measurements recorded (sampling rate 360 s^{-1}) during the first 10 s of the reaction.

Analyses. For amino acid analysis, samples were hydrolyzed in constant boiling 6 M HCl for 24 h at 108 °C. The dried

[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); CM, carboxymethyl; Con A, concanavalin A; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G; LPO, bovine lactoperoxidase; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); NbsH, 5-mercapto-2-nitrobenzoic acid; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPO, human salivary peroxidase; TMBZ, 3,3′,5,5′-tetramethylbenzidine; Tris-HCl, tris(hydroxymethyl)aminomethane.

Table I: Purification of Salivary Peroxidase

step	total protein (mg)	total NbsSCN act. (mmol/min)	sp act. (mmol min^{-1} mg^{-1})	x-fold purifcn (based on sp act.)	recovery (based on total act.) (%)	recovery (based on total protein) (%)
PBS–EDTA saliva	4371.7	801	0.18			
immunoaffinity chromatography	85.8	769	8.96	50	96.0	2.0
CM-Sepharose Fast Flow	24.6	740	30.1	167	92.4	0.56
Blue Sepharose I	14.0	547	39.1	217	68.3	0.32
Blue Sepharose II	2.25	333	148	822	41.6	0.05

hydrolysates were analyzed according to the method of Butler et al. (1977) using a Beckman 121 M amino acid analyzer.

Quantitative analysis of the neutral sugar content of SPO and LPO was carried out according to the method described by Hjerpe et al. (1983). Samples were methanolyzed at 100 °C for 50 h using 1 M HCl in dry methanol and chromatographed on a CP MicroSpher C_{18} column (100 × 4.6 mm i.d.) or a Hypersil ODS column (250 × 4.6 mm i.d.) (Chrompack, Middelburg, The Netherlands).

Hexosamines were quantitated as described by Hjerpe et al. (1980). After hydrolysis with 8 M HCl at 100 °C for 3.5 h, the samples were dansylated and subsequently purified on a Sep-Pak C_{18} cartridge (Waters Associates). The hexosamine derivatives were separated on a Hypersil ODS column. Dansylated tris(hydroxymethyl)aminomethane was used as an internal standard.

Salivary peroxidase and LPO were deglycosylated by using *N*-glycanase (Genzyme, Boston, MA) as described by Plummer et al. (1984). Human transferrin served as control to determine that the *N*-glycanase enzyme was active. The deglycosylated samples were analyzed on 5–15% SDS–PAGE as described below.

Molecular Weight Determinations and Evaluation of Homogeneity. Slab gel electrophoresis, using 1.5-mm gels, was performed essentially as described by Laemmli (1970) and modified by Butler et al. (1981). All gels were stained with Coomassie Brilliant Blue R. Molecular weight standards included phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

The behavior and the molecular weight of the proteins in SDS–PAGE were tested by repeated electrophoresis in a series of different gel concentrations (7.5%, 10%, 12.5%, 15%, 17.5%, and 20%). The data were analyzed as proposed by Ferguson (1964) and as modified by us. A constant ratio of *N*,*N*′-methylenebis(acrylamide) was used for all gels, and the gels were electrophoresed in the same manner as the gradient gel.

Mobilities of individual proteins were determined by measuring the distance from the point of origin to the leading edge of the band. The mobility measurements for each protein in a series of gels were then analyzed in terms of the relationship

$$M = (M_0) \exp(-K_r T) \quad (1)$$

where M is the mobility, M_0 is the free mobility, K_r is the retardation coefficient, and T is the gel percent (Mantle, 1978). For each standard and for SPO and LPO, values of M_0 and K_r were estimated by nonlinear least-squares regression analysis (Ralston, 1983). The following empirical relationship was assumed between molecular weight (M_w) and M_0 and K_r

$$M_w = (P_1)K_r + (P_2/M_0) + P_3 \quad (2)$$

where P_1, P_2, and P_3 are arbitrary parameters. To our knowledge, this relationship has not been previously described. The rationale for it is given under Results and Discussion. Estimates of P_1, P_2, and P_3 were obtained by nonlinear regression analysis of the M_w, K_r, and M_0 data for the set of molecular weight standards (Ralston, 1983). The molecular weights of SPO and LPO were calculated by substituting these estimates of P_1, P_2, and P_3 together with the previously obtained values of K_r and M_0 for the peroxidases into eq 2.

Detection of Peroxidase Activity on SDS–PAGE. The gels were stained for peroxidase activity with TMBZ and hydrogen peroxide as described by Thomas et al. (1976). The enzyme preparations were electrophoresed as described above and the gels rinsed briefly in quartz distilled water. Freshly prepared TMBZ solution in methanol (6.3 mM) was mixed with 0.25 M sodium acetate buffer, pH 5.0, immediately before use. The gels were immersed in the TMBZ solution and incubated in the dark at room temperature for 45 min, after which hydrogen peroxide was added. The peroxidase bands appeared blue on the gel, and the color was stable up to 12 months, even after the gels were dried.

Results and Discussion

The peroxidase activity in the individual samples of human parotid saliva from one of the donors varied from 840 tc 1616 μmol/min (mean, 1254 μmol/min) as analyzed by the NbsSCN assay. The protein concentration of the saliva samples varied from 6.1 to 9.5 mg/mL (mean, 7.0 mg/mL).

The purification scheme involved sequential passage through an immunoaffinity column, cation-exchange chromatography, and repeated affinity chromatography on Blue Sepharose CL-6B. A summary of the purification of SPO is presented in Table I. The purification fold was 822, and the final yield of the SPO enzyme was 42% of the original enzyme activity, which represented 0.05% of the original protein. An insignificant portion of the protein was lost during the procedures used for the purification of the enzyme, since 96.3% of the total protein could be accounted for (results not shown).

The immunoaffinity chromatography resulted in a 50-fold purification of the enzyme (Table I). Approximately 96% of the applied protein was eluted in the wash fractions, and these fractions did not contain any peroxidase enzyme activity (Figure 1A). Salivary peroxidase was eluted from the column with PBS buffer containing 3.0 M NaSCN. When this fraction was analyzed on SDS–PAGE, SPO was the major protein. However, several minor contaminating proteins were present.

The second purification step involved cation-exchange chromatography (Figure 1B), which resulted in an enzyme preparation with only one contaminating protein with an apparent molecular weight of 45 000 when analyzed on SDS–PAGE.

Further purification of the peroxidase enzyme was achieved by chromatography on Blue Sepharose CL-6B. This step resulted in a 217-fold purification. Rechromatography on the same column and under identical conditions resulted in a preparation with sufficient purity for subsequent analysis (Figure 1C).

The final product (2.25 mg of protein) from the chromatographic procedures had an enzyme activity of 333 mmol/

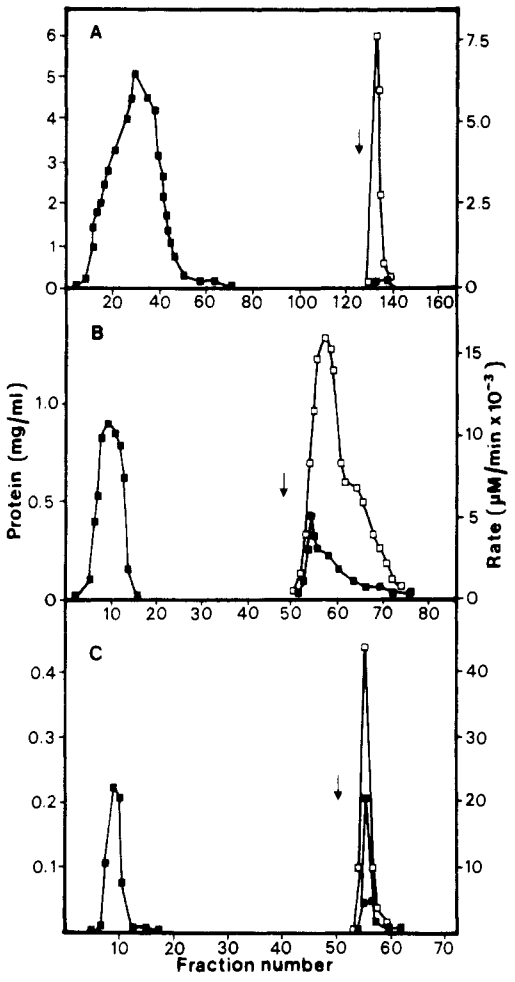

FIGURE 1: Chromatographic purification of human SPO. (Panel A) Immunoaffinity chromatography. The arrow indicates elution of enzyme containing fractions using 3.0 M NaSCN in PBS, pH 7.0. (Panel B) Cation-exchange chromatography on a CM-Sepharose Fast Flow column (2.5 × 10 cm). The arrow indicates elution of SPO using 0.6 M NaOAc at pH 7.0. (Panel C) Affinity chromatography on a column of Blue Sepharose CL-6B. Salivary peroxidase was eluted with 0.6 M NaCl in 0.05 M Tris-HCl buffer, pH 7.4. The arrow indicates the start of the elution. The fractions were monitored for the absorbance at 280 nm, and the protein content (■) was determined by the method described by Bradford et al. (1976) using bovine serum albumin as a standard. The SPO activity (□) was determined by the NbsSCN assay as described under Experimental Procedures.

min. The values presented in Table I for the last purification step are the added values for the protein and peroxidase content. The fractions with the highest enzyme activity and the highest ratio of A_{412nm}/A_{280nm} (0.81) were pooled and had a specific activity of 0.179 mmol of Nbs oxidized per min per mg of protein. These fractions represented a 992-fold purification of the enzyme. They were used for the spectral scan, amino acid, and carbohydrate analyses, for the molecular weight estimates, and for determination of other properties of the enzyme.

The purity of the SPO preparation was estimated by the ratio A_{412nm}/A_{280nm} (Figure 2). This ratio (purity index) has been used traditionally as a measure of purity of peroxidase preparations. The purity index for the purified SPO was 0.81, which can be compared to a value of 0.68 reported by Slowey et al. (1968). Thus, our SPO preparation had the highest purity index ever reported for SPO. The earlier report (Slowey et al., 1968) of 1026-fold purification of SPO may seem in conflict with our results of a 992-fold purification of the enzyme with a higher purity index. These differences are not significant in view of the basis upon which the calculations

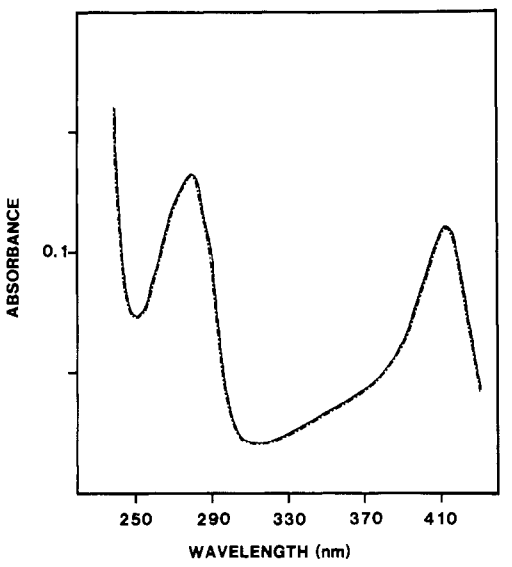

FIGURE 2: Absorption spectrum of human SPO (—) and bovine lactoperoxidase (- - -) in 0.05 M Tris-HCl containing 0.6 M NaCl, pH 7.4, at 25 °C. The protein concentration was 88.6 μg/mL and A_{412nm}/A_{280nm} = 0.81 for both preparations.

Table II: Amino Acid Composition

	residues per 1000 residues	
	human salivary peroxidase	bovine lactoperoxidase[a]
aspartic acid	118	127 ± 8
threonine	49	50 ± 4
serine	77	60 ± 3
glutamic acid	105	107 ± 1
proline	97	80 ± 7
glycine	77	77 ± 4
alanine	74	69 ± 3
cysteine	16	15 ± 7
valine	38	40 ± 7
methionine	5	16 ± 7
isoleucine	31	32 ± 3
leucine	117	113 ± 2
tyrosine	22	27 ± 1
phenylalanine	50	52 ± 1
lysine	50	55 ± 2
histidine	18	23 ± 1
arginine	57	61 ± 2
tryptophan	ND[b]	ND[b]

[a] Mean ± SD for three determinations. [b] ND = not determined.

were made. The purification fold will depend upon the protein concentration in the original saliva sample used in the initial purification step, and this concentration will vary from preparation to preparation. Furthermore, Slowey et al. used *o*-dianisidine as a substrate for peroxidase assays. This substrate would be affected by physiological concentrations of SCN^- in saliva and would result in underestimation of the enzyme activity in the original sample (Mansson-Rahemtulla et al., 1986). The purification procedure described above was repeated twice with the saliva samples from the other two donors, and no significant differences were observed among the three samples.

The amino acid composition of SPO is shown in Table II. For comparative purposes, amino acid analysis of commercially obtained LPO (purity index A_{412nm}/A_{280nm} = 0.92) was also performed under identical conditions. The results obtained for LPO are consistent with previously published analyses (Morrison & Steele, 1968; Carlstrom, 1969). As can be seen in Table II, the overall amino acid composition of the two enzymes is similar. However, salivary peroxidase contains

Table III: Carbohydrate Analysis[a]

	human salivary peroxidase	bovine lactoperoxidase
neutral sugar total	45.8	70.7
galactose	10.4	4.0
mannose	15.4	59.2
fucose	14.2	1.9
glucose	5.8	5.6
glucosamine	58.4	27.0
galactosamine	27.5	9.0

[a] Micrograms of carbohydrate per milligram of dry weight of sample.

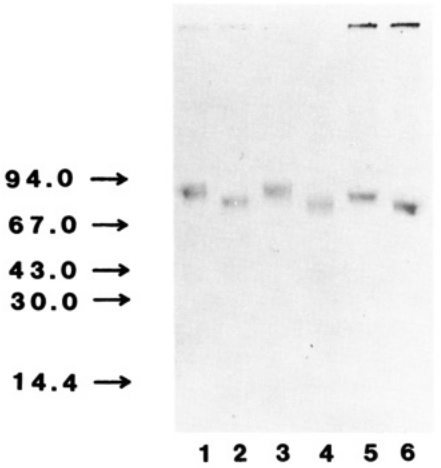

FIGURE 3: Analysis of SPO (lanes 1 and 2), bovine LPO (lanes 3 and 4), and human transferrin (lanes 5 and 6) on SDS–PAGE after treatment with *N*-glycanase. The gels were stained with Coomassie. Lanes 1, 3, and 5 represent samples that have been incubated with *N*-glycanase at 30 °C for 16 h. Lanes 2, 4, and 6 have been treated identically, but the *N*-glycanase was substituted with the same volume of glycerol.

more proline and serine than does LPO. It is possible that the higher content of proline in SPO could be due to trace amounts of proline-rich proteins. However, the proline-enriched proteins are also rich in glycine and glutamic acid compared to other proteins. Thus, contamination of the SPO by a proline-rich protein should produce higher amounts of these latter amino acids compared to those produced by LPO. However, our analyses showed that the glycine and glutamic acid contents of LPO and SPO are the same. Taken together, these data suggest that the higher content of proline in the salivary enzyme is an intrinsic property of the molecule.

The similarities in the protein composition with regard to aromatic amino acids were confirmed by the UV–vis spectrum of the two proteins, which were identical with well-defined peaks at 280 nm. The hemoprotein nature of the two enzymes was shown by the absorption peak at 412 nm (Figure 2).

The two enzymes differed in carbohydrate content and composition. SPO contained 12.7% and LPO 10.7% total carbohydrates. In SPO, 4.6% of the carbohydrates were neutral monosaccharides, and the corresponding figure for LPO was 7% (Table III). The value for LPO is in good agreement with other reports (Robauts et al., 1967; Morrison & Steele, 1968; Carlstrom, 1969). Mannose, fucose, and galactose were found in SPO in the molar ratio 1.5:1.5:1.0, while the ratio in LPO was 14.9:0.5:1.0. The oligosaccharides in both enzymes were susceptible to the specific enzyme *N*-glycanase, demonstrating the N-linked oligosaccharide nature of these carbohydrate side chains (Figure 3). The ratio of glucosamine to galactosamine was 2:1 in SPO and 3:1 in LPO. The relatively large amounts of glucosamine and mannose in

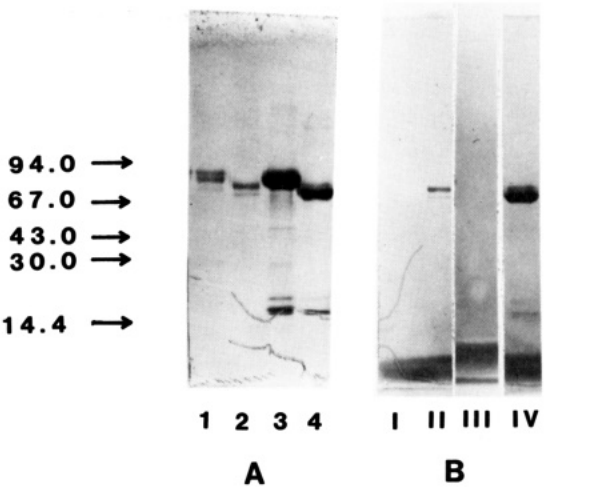

FIGURE 4: SDS–PAGE analysis of purified human SPO from Figure 1 and commercially obtained bovine lactoperoxidase. Fractions with the highest purity index (A_{412nm}/A_{280nm} = 0.78–0.81) were pooled and analyzed on 5–15% gradient gels. (Panel A) Commassie stain: (lane 1) reduced SP (100 μg); (lane 2) nonreduced SPO (100 μg); (lane 3) reduced lactoperoxidase (25 μg); (lane 4) nonreduced lactoperoxidase (25 μg). (Panel B) Stain for peroxidase activity using TMBZ as a substrate. The details for the procedure are given under Experimental Procedures. Lanes I–IV correspond to lanes 1–4 in panel A.

both enzymes indicate that the majority of the oligosaccharides are of high-mannose type. The small amounts of glucose detected in both the preparations by the sensitive HPLC method may be contaminants. Further studies are required to substantiate the presence of glucose as a covalently bound component of these enzymes.

Salivary peroxidase appeared on SDS–PAGE, 5–15% gradient gels, as three bands. Two of the bands migrated close together and were both of higher molecular weight than LPO. The third band had an apparent molecular weight of 280 000. All three bands stained positively for both Coomassie and enzyme activity (Figure 4).

Because SDS–PAGE did not result in dissociation of the high molecular weight band, this band may be a covalently bonded aggregate of SPO molecules. Aggregation of SPO has previously been reported (Makinen & Tenovuo, 1976; Tenovuo, 1981). These and other investigators (Iwamoto, 1968, 1972; Makinen & Tenovuo, 1976; Azen, 1977, 1985; Tenovuo, 1981) have also described heterogeneity of the SPO molecule, in agreement with our results. However, Tenovuo (1981) reported that the heterogeneity of the molecule could be observed only after isoelectric focusing. Azen (1977, 1985) described three forms of SPO, two of which, SAPX 2 and SAPX 3, were always found in association with acidic proline-rich proteins. These complexed forms of SPO could be dissociated to a third and most common form, SAPX 1, only after reduction with 2-mercaptoethanol. Our preparation of SPO cannot be directly compared with Azen's SAPX since he used unfractionated parotid saliva and our preparation is the highly purified enzyme. We found that SPO continues to appear as two bands on SDS–PAGE after reduction with 2-mercaptoethanol and that both bands show a decreased migration into the gel. We were not able to detect any enzyme activity on the gels after reduction. Azen reported both increased mobility and retention of enzyme activity after treatment with 2-mercaptoethanol. However, he did not include SDS in the electrophoretic system, and the concentration of 2-mercaptoethanol which he employed was significantly lower than that used in the present study.

Table IV: Molecular Weight Determination

molecular weights		Ferguson parameters[a]	
actual	calcd[b]	retardation coeff (gel %)$^{-1}$	free mobility (M_0) (mm)
94 000	92 800	0.198	24.7
67 000	69 300	0.178	28.1
43 000	42 000	0.155	33.5
30 000	30 000	0.137	33.7
human salivary peroxidase	77 200	0.195	29.1
bovine lactoperoxidase	75 100	0.196	30.3

[a] Values of the parameters K_r and M_0 were determined by fitting mobility data to eq 1 as described in the text. [b] The molecular weights of the standards together with the values of K_r and M_0 were used together with eq 2 to obtain estimates of P_1, P_2, and P_3. By use of these latter estimates of parameter values, molecular weights were calculated from eq 2.

Table V: Consistency and Accuracy of the Modified Ferguson Analysis

molecular weights		Ferguson parameters[a]	
actual	calcd[b]	retardation coeff (gel %)$^{-1}$	free mobility (M_0) (mm)
94 000	94 600 ± 1700	0.194 ± 0.022	23.3 ± 4.5
67 000	65 000 ± 3300	0.177 ± 0.005	29.9 ± 2.3
43 000	43 200 ± 2100	0.160 ± 0.005	36.6 ± 2.7
30 000	30 800 ± 1200	0.144 ± 0.006	37.8 ± 3.6

[a] Determined by fitting the mobility data to eq 1. Value ± SD. [b] Calculated by fitting the Ferguson parameters to eq 2. Mean ± SD for three determinations.

Reduction of SPO and LPO with 2-mercaptoethanol resulted in a decreased migration into the gel, corresponding to an increase in molecular weight of 16 000 for SPO and 14 000 for LPO (Figure 4A). This change in electrophoretic mobility indicated that disulfide linkages had been reduced, resulting in more extended forms of the molecules.

The molecular weights of SPO and LPO were determined according to the methods suggested by Ferguson (1964) and calculated as described under Experimental Procedures. This analysis typically utilizes only the estimates of K_r obtained by fitting eq 1 to the mobility data. However, the free mobility is obviously a significant parameter in electrophoretic experiments. In order to utilize its value in estimating molecular weights, a quantitative relationship between M_w, K_r, and M_0 is required. The theoretical basis for analysis of electrophoretic data is complex (Rodbard & Chrambach, 1970). We used a purely empirical approach and reasoned that molecular weights calculated from mobility data should be directly proportional to K_r and inversely proportional to M_0. We postulated eq 2 as a simple relationship that incorporated these assumptions and tested the equation using the mobility data for the standards. The results in Table IV show that there is satisfactory agreement between the actual values of the molecular weights of the standards and the predicted values based on eq 2. The retardation coefficient and the free mobility for the molecular weight standards were determined in three separate experiments. The results in Table V show that the method is accurate and reproducible. For SPO, the molecular weight was determined for the middle, most prominent band. Resolution into three different bands was not always obtained and depended upon polyacrylamide concentration.

The mobility data measured for the molecular weight standards and for SPO and LPO were very consistent with eq 1. Equation 2 gave a very good fit to the estimates of the retardation coefficient (K_r) and the free mobility (M_0) obtained for the mobility data. By use of the values of arbitrary parameters P_1, P_2, and P_3 obtained from analysis of the data in Table IV, the following molecular weights were calculated from eq 2: 77 000 ± 2000 for SPO and 75 000 ± 2000 for LPO. This is in good agreement with the finding of a molecular weight of 73 000 when salivary peroxidase was fractionated by gel filtration chromatography on a column of Sepharcryl S-200 Superfine that was equilibrated with well-known protein standards (results not shown). The actual molecular weight of lactoperoxidase determined from amino acid composition is in the range of 77 000–79 000 (Carlstrom, 1969; Sievers, 1980; Paul, 1985).

The enzyme activity in parotid saliva was stable for several days when stored at 4 °C. No loss of enzyme activity was observed when saliva was stored for 6 months at −20 °C. The partially and highly purified preparations of SPO showed no loss of activity when stored in PBS, pH 7.0, or in 0.05 M Tris-HCl containing 0.5 M NaCl, pH 7.4, at −20 °C for 6 months.

Both SPO and LPO (6 μg/mL in 0.01 M phosphate buffer, pH 7.0, containing 0.1% gelatin) were relatively stable toward thermal denaturation. After incubation for 48 h at 37 °C, both enzymes retained 57% of the initial activity. At 50 °C, 32% of the SPO and 42% of the LPO activity remained after 48 h. Both enzymes lost all activity after 5 h at 65 °C.

In order to determine specific ABTS activity, peak fractions of SPO from three separate final elutions from Blue Sepharose were used. The fractions had purity indices of 0.78–0.81. For the commercial sample of LPO used, the value was 0.92. Protein concentrations were determined by assuming A_{280nm} = 14.9 for a 1% solution of both enzymes (Carlstrom, 1965). The ABTS activity of the solutions was measured and divided by the protein concentration. The means and standard deviations from three separate experiments were SPO = 341 ± 17 millunits/μg and LPO = 65 ± 5 milliunits/μg.

The effects of the classical peroxidase inhibitors sodium cyanide and sodium azide were studied by incubating SPO and LPO (6 μg/mL) with varying concentrations (0.5–20 mM) of the inhibitors for 30 min at room temperature. After the incubation period, the remaining enzyme activity (E) was measured by the ABTS assay. We found that E decreased exponentially with increasing concentrations of inhibitor (I). We interpreted these (E, I) curves as follows. Assuming that the loss of enzyme activity after the incubation period ($-dE$) is directly proportional to the increment of inhibitor added (dI) and is first order with respect to E gives the relationship

$$-dE = kE\,dI \tag{3}$$

where k is the apparent rate constants for inhibition. If the enzyme activity after incubation in the absence of inhibitor is equal to E_0, integration of eq 3 gives

$$-\ln E/E_0 = kI \tag{4}$$

If $I_{1/2}$ is the inhibitor concentration that reduces E_0 by 1/2, then eq 4 may be written

$$E/E_0 = 2^{-I/I_{1/2}} \tag{5}$$

Values of $I_{1/2}$ were estimated by fitting the data to eq 5 using nonlinear least-squares analysis. SPO was much more sensitive to azide than was LPO. The $I_{1/2}$ were 0.12 ± 0.01 mM (n = 6) for SPO and 2.4 ± 0.9 mM (n = 6) for LPO. The sensitivity to cyanide was comparable for the two enzymes. The corresponding $I_{1/2}$ estimates for cyanide were 2.3 ± 0.5 mM (n = 6) for SPO and 3.2 ± 1.2 mM (n = 6) for LPO. Both azide and cyanide block the oxidation of the peroxidase

enzyme to the reactive intermediate compound I by binding to the iron atom in the prosthetic group (Paul & Ohlsson, 1985).

In summary, our results show that LPO and SPO are similar in amino acid composition, ultraviolet and visible spectrum, reaction with cyanide, susceptibility to reduction by mercaptoethanol, and thermal stability. However, SPO contains a significant greater number of proline residues than does LPO and is more sensitive to azide than is LPO. The two enzymes also differ in carbohydrate composition and in specific peroxidase enzyme activity on the basis of on ABTS analysis. These differences suggest that SPO and LPO differ in secondary and tertiary structure, and this suggestion is supported by the quantitative differences in kinetic properties that have been observed (Pruitt et al., 1988).

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