Identification, Purification, and Characterization of a Non-heme Lactoperoxidase in Bovine Milk*

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A purification procedure for a protein related to lactoperoxidase devoid of the heme prosthetic group under conditions also yielding enzymatically active lactoperoxidase is described. These two forms were separated from bovine milk according to their respective behaviors on cation exchange. Lactoperoxidase and non-heme lactoperoxidase had the same apparent molecular weight in the denatured (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and native form (velocity sedimentation on sucrose gradient) about 85,000; but unlike lactoperoxidase, non-heme lactoperoxidase was devoid of light absorption properties in the Soret region and of enzyme activity. Lactoperoxidase and non-heme lactoperoxidase contained a similar amount of carbohydrate and gave very similar peptide maps after limited proteolysis by subtilisin or trypsin. The two forms appeared to be immunologically related since they gave a single line in immunodiffusion using anti-lactoperoxidase antibodies and since 125I-labeled non-heme lactoperoxidase and 125I-labeled lactoperoxidase reacted with anti-lactoperoxidase antibodies in radioimmunoassay.

Lactoperoxidase and nonheme lactoperoxidase were compared in their ability to interact with dityrosine and tubulin. (Rousset, B., and Wolff, J. (1980) J. Biol. Chem. 255, 2514-2523). 125I-labeled dityrosine bound specifically to lactoperoxidase. No detectable binding has been observed with nonheme lactoperoxidase. In contrast, lactoperoxidase and nonheme lactoperoxidase coupled to an insoluble matrix were able to bind rat brain tubulin, indicating that both forms of lactoperoxidase can be used for an affinity chromatography purification procedure of brain tubulin. Non-heme lactoperoxidase was found in milk from several origins, cow, goat, sheep, and human. In bovine milk, lactoperoxidase and non-heme lactoperoxidase were found in comparable amounts.

Lactoperoxidase is a hemoprotein present in milk, tears, and saliva (1). First attempts to purify this enzyme date back to 1925 using ammonium sulfate precipitation. Since then, various chromatographic techniques based on ion exchange procedures have been employed (2-5). More recently, an affinity chromatography method has been developed (6); this method is based on the interaction of lactoperoxidase with thiols and diityrosine. It is now well established that lactoperoxidase is a basic protein consisting of one polypeptide chain (7) of 80,000-85,000 molecular weight and containing 8-10% carbohydrate (8). The prosthetic group of lactoperoxidase is related to protoporphyrin IX and may be covalently linked to the protein (9) through an ester linkage involving a carboxyl group of the protein and a hydroxyl group of the heme (1). During the course of the purification of lactoperoxidase by cation exchange chromatography according to Paul et al. (5) we have identified a basic protein in whey which displays the biochemical characteristics of a non-heme lactoperoxidase. In this paper we describe its purification and identification and some of its biochemical properties.

EXPERIMENTAL PROCEDURES

Materials

Lactoperoxidase was obtained from Boehringer Mannheim. Monoiodotyrosine, SDS,5 GTP, Mes, and rennet (21 units/mg; 1 unit will coagulate 10 ml of milk per min at 30°C) were from Sigma. Amberlite CG-50 was purchased from Serva, Affi-Gel 10 from Bio-Rad, and Iobeads from Technicon. Products for electrophoresis and molecular weight markers were from Bio-Rad, chloramine-T was obtained from Merck, and Na125I was from Amersham Corp. Fresh bovine milk was obtained by courtesy of Vivalp, S.A.-Lyon. Sheep and goat milks were obtained directly at the farm. Fresh milk (especially bovine milk) used throughout the study contained less than 2 x 109 bacteria per ml.

Lactoperoxidase Purification Procedure

In the initial step, casein was removed from fresh raw skim bovine milk. After addition of 10 mg of rennet per liter, the milk was stirred at room temperature until coagulation occurred. All subsequent steps were carried out at 4°C. The coagulated milk was centrifuged for 15 min at 2,600 x g in a Sorvall HS4 rotor. To each liter of whey were added 40 ml of wet Amberlite CG-50 (NH+, form) previously washed with water and 50 mM sodium acetate. Ammonium sulfate was removed by gel filtration on Sephadex G-25 and eluted by a gradient composed of 25 ml each of 100 mM sodium acetate (1 liter and 2 liters each per liter of whey, respectively). The resin was then transferred to a Büchner funnel and washed with water and 50 mM sodium acetate (1 liter and 2 liters each per liter of whey, respectively). The resin was then transferred to a column (1.5 x 50 cm) and elution was carried out in two steps using 0.5 M sodium acetate and 1.0 M sodium acetate. The flow rate was 2-5 ml/min.

To the green-colored material eluted with 0.5 M sodium acetate containing hemic lactoperoxidase were gradually added over a period of 30 min and with constant mixing 500 g of solid ammonium sulfate per liter of solution. The precipitate was then sedimented by centrifugation at 3,000 x g for 30 min and suspended in 100 mM sodium acetate. Ammonium sulfate was removed by gel filtration on Sephadex G-25 equilibrated in 100 mM sodium acetate. Lactoperoxidase was further purified by a second filtration on the columns of Amberlite CG-50 and eluted by a gradient composed of 25 ml each of 100 mM and 1.0 M sodium acetate. This second column allowed removal of residual non-heme lactoperoxidase from lactoperoxidase. Lactoperoxidase obtained after this second purification step was then used for...

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5 The abbreviations used are: SDS, sodium dodecyl sulfate; Mes, 2-(N-morpholino)ethanesulfonic acid; GTP, guanosine 5'-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
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further experiments. Protein eluted from the column using 1 M sodium acetate (stepwise elution) or between 0.6 and 1.0 M gradient elution containing non-heme lactoperoxidase was first diluted to twice its initial volume by adding water and precipitated by addition of ammonium sulfate over a period of 30 min with constant stirring as described above. Both lactoperoxidase and non-heme lactoperoxidase were stored at 4 °C in 3.2 M ammonium sulfate and equilibrated with the appropriate buffer by gel filtration on Sephadex G-25 before use.

Preparation of Antisera

Two male rabbits (albino hybrids) were injected on days 1 and 23 with 2 ml of a suspension containing 1 mg of pure lactoperoxidase and 1 ml of complete Freund's adjuvant. Rabbits were bled on day 28 and sera were stored at −20 °C.

Labeling of Lactoperoxidase

Lactoperoxidase and non-heme lactoperoxidase were iodinated by chemical oxidation. The reaction mixture (30 μl) contained 20 μg of lactoperoxidase or non-heme lactoperoxidase, 1 mM of carrier-free 125I- Na, 40 μg of chloramine-T in 0.2 M sodium phosphate buffer, pH 7.2. The reaction was stopped after 1 min by addition of 25 μl of sodium metabisulfite (2.4 mg/ml), and the solution was supplemented with 1 mg of bovine serum albumin. The labeled protein was separated from [125I]iodide by gel filtration on Sephadex G-25 equilibrated in 10 mM sodium phosphate, 154 mM NaCl, bovine serum albumin, 5 mg/ml, pH 7.4. [125I]-labeled proteins (specific radioactivity, ≈80 μCi/μg) were stored in liquid nitrogen.

Preparation of [125I]Diiodotyrosine

[125I]Diiodotyrosine was obtained by iodination of monoiodotyrosine with 125I in the presence of chloramine-T. The reaction mixture contained 14 μM monoiodotyrosine, 3.7 μM 125I (specific radioactivity, 1920 Ci/mmol) and 5 mM chloramine-T in 0.2 M sodium phosphate buffer, pH 7.40. The reaction was allowed to proceed at room temperature for 2 min and then stopped by the addition of 0.66 volume of 130 mM sodium metabisulfite. The solution was then incubated for 2 min with an anion exchange resin (Iobeads) to remove unreacted iodide. Identification of reaction products was performed by paper chromatography carried out by the ascending technique in 1-butanol-acetic acid-water (4:1:5) according to Ref. 10 using iodide, monoiodotyrosine, and diiodotyrosine as reference compounds.

Binding Experiments

Diiodotyrosine—Binding of [125I]diiodotyrosine to lactoperoxidase was achieved by incubation at 0 °C for 5 min. The reaction mixture was passed over a 75-μl column of Amberlite CG-50 to separate free from bound [125I]-labeled diiodotyrosine. The resin in washed with buffer until the effluent no longer contained radioactivity. Diiodotyrosine was not retained by the cation exchanger. Bound [125I]diiodotyrosine was measured by counting the radioactivity of the column in a well-type Packard γ counter and subtracted for blanks.

Tubulin—Interaction of tubulin with both forms of lactoperoxidase was studied using an affinity chromatography procedure previously described (11). Lactoperoxidase and non-heme lactoperoxidase were coupled to Affi-Gel 10 through a succinylated aminosilanyl spacer. Affi-Gel 10 prewashed with 10 volumes of water and 2.5 volumes of Mes, 25 mM buffer, pH 6.40, was mixed with solutions of lactoperoxidase or non-heme lactoperoxidase (2 mg/ml) at a ratio of 1 ml of gel per ml of ligand solution. The suspension was tumbled for 3 h and then reacted with 0.1 volume of 1 M ethanolamine at pH 8.0 for 1 h at 4 °C. Gels were then washed with buffer containing 1 M NaCl and equilibrated with the buffer used for the affinity chromatography.

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed on slab gels with a 4.5% stacking gel and a 0% running gel as previously described (11). Proteins were stained using 0.1% Coomassie brilliant blue in 50% (v/v) trichloroacetic acid. For glycoprotein staining, gels were washed overnight in a solution containing 40% methanol and 7% acetic acid and incubated in a 1% periodic acid, 7% acetic acid solution for 60 min at 4 °C in the dark. Gels were then incubated in the Schiff’s reagent for 60 min at 4 °C in the dark and washed in 1% sodium metabisulfite, 0.1 N hydrochloric acid.

Peptide Mapping

Protein hydrolysis and peptide analysis were performed according to Cleveland et al. (12). Proteins (1–2 mg/ml) in 0.125 M Tris/HCl, 0.5% SDS, 10% glycerol, pH 6.8, were boiled for 2 min at 100 °C. Aliquots of 100 μl of protein solution were incubated with 10 μl of subtilisin (0.25 mg/ml or trypsin (2 mg/ml) for 30 min at 37 °C. Following addition of 25 μl of 10% SDS and 12.5 μl of 2 mercaptoethanol, proteolysis was stopped by boiling the samples for 2 min. About 30 μl of each sample were analyzed on 10% acrylamide gel. Staining was performed using Coomassie blue.

Other Analytical Methods

Proteins were determined by the method of Bradford (13) using the Bio-Rad protein reagent and bovine serum albumin as standard or by absorbance measurements at 280 and/or 412 nm.

Peroxidase activity was assayed using iodide and H2O2 according to Ref. 14. The rate of oxidation of iodide was followed at 350 nm. The assay was carried out at 20–22 °C in 1.0-cm light path cuvette containing 3 ml of a solution which was 33 mM sodium phosphate, pH 7.0, 5 mM sodium iodide, and 0.15 mM H2O2. The amount of enzyme which gave an absorbence change of 0.06 per min was taken as 1 millunit. Spectrophotometric measurements were performed at room temperature in a 24 K Beckman spectrophotometer using 1-cm light path cuvettes.

Bacterial counts were made after 24 and 48 h of culture on gelose at 30 °C. Each sample was tested at several dilutions ranging from 10−2 to 10−7. Bacterial counts have been made according to routine procedures for detection of mesophile organisms in the Laboratoire de Bactériologie, Hôpital Jules Cournon, Lyon.

Double immunodiffusion was carried out in immunodiffusion discs (Miles, Paris). The antigens was contained in 5-mm diameter wells around the central well at a distance of 8 mm. Discs were kept at 4 °C and examined after 5 to 10 days.

RESULTS

Purification of Lactoperoxidase-related Proteins

Elution of whey proteins adsorbed on the cation exchange resin was monitored by absorbance measurements at 280 and 412 nm (Fig. 1A). The protein fraction removed from the column by 0.5 M sodium acetate (Fraction A) had an average A412/A280 ratio of 0.4 (average of 5 preparations), whereas proteins eluted by 1 M sodium acetate (Fraction B) had an A412/A280 ratio lower than 0.03. SDS-polyacrylamide gel electrophoresis (Fig. 1B) showed that fractions A and B actually contain a major protein band having the same apparent molecular weight of 85,000 and a molecular weight identical with that of lactoperoxidase obtained from Boehringer Mannheim. The Mw = 85,000 protein represents 90% or more of the total protein in each case. Protein molecules of fractions A and B differ by their absorption properties in the Soret region. The similarity in molecular weight and the difference in A412 led us to consider that protein of fractions A and B could correspond to the heme and non-heme forms of lactoperoxidase, respectively. Compared to commercial lactoperoxidase (Boehringer Mannheim), the protein of fraction A had a rather low A412/A280. When protein of fraction A was chromatographed again on the cation exchanger and eluted with a linear gradient (0.1 to 1.0 M sodium acetate) two protein peaks were obtained, fractions C and D (Fig. 1C). Fraction C had an A412/A280 ratio of about 0.8 which corresponds to that of the purest commercial preparation of lactoperoxidase. The protein of fraction D did not exhibit absorption at 412 nm.

Materials of fractions C and D are again similar in terms of molecular weight determined by SDS-polyacrylamide gel electrophoresis (results not shown). Materials of fractions B and C were used as non-heme lactoperoxidase and lactoperoxidase in the following experiments.

In order to examine whether the bacterial status of the milk influenced the formation and/or the recovery of non-heme lactoperoxidase, the following experiments were performed.
Fresh milk was submitted to 6-h treatments at 4 °C (control), at 30 °C (to promote bacterial growth), and at 30 °C in the presence of antibiotics. Bacterial counts were performed at the end of the treatment and on the corresponding whey preparations. Quantitation of non-heme lactoperoxidase (fraction B) in each sample of whey was conducted as described under “Materials and Methods.” Results are presented in Table I. Regardless of bacterial contamination, similar amounts of non-heme lactoperoxidase were extracted. The isolation procedure from whey by cation exchange chromatography yielded more non-heme lactoperoxidase than lactoperoxidase. Lactoperoxidase and the non-heme form sedimented as symmetrical peaks which superimposed. As compared to marker proteins, the apparent molecular weight of the two proteins (in the native form) was about 85,000 (Fig. 2, inset). Lactoperoxidase and the non-heme form are composed of a single polypeptide chain since the same apparent molecular weight was obtained under nondenaturing and denaturing conditions. This has already been reported for lactoperoxidase by Sievers (7).

Characterization of Non-heme Lactoperoxidase

The protein fraction called “non-heme lactoperoxidase” had the same apparent molecular weight as lactoperoxidase on SDS-polyacrylamide gel electrophoresis (Fig. 1B). Comparison of the molecular weights of the two forms was further defined by velocity sedimentation analysis on 5–17% sucrose gradients (Fig. 2). Lactoperoxidase and non-heme lactoperoxidase sedimented as symmetrical peaks which superimposed. As compared to marker proteins, the apparent molecular weight of the two proteins (in the native form) was about 85,000 (Fig. 2, inset). Lactoperoxidase and the non-heme form are composed of a single polypeptide chain since the same apparent molecular weight was obtained under nondenaturing and denaturing conditions. This has already been reported for lactoperoxidase by Sievers (7).

Fig. 3 shows the ultraviolet-visible absorption spectra of the two forms of the protein. Lactoperoxidase possesses a Soret band with an absorption maximum at 412 nm. Non-heme lactoperoxidase did not exhibit significant absorption activity in the 300- to 500-nm region; this indicates that the prosthetic group protoporphyrin IX was not present (9). The molar absorption coefficient at 280 nm for the two proteins are similar, ε85,000 M⁻¹ cm⁻¹.

The peroxidase activity (oxidation of iodide) of the two protein fractions was different. The enzyme activity of lactoperoxidase was between 50 and 70 units/mg whereas that of non-heme lactoperoxidase was less than 0.5 units/mg.

Lactoperoxidase and non-heme lactoperoxidase were both

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**Table I**

| Treatment of the milk | Bacterial counts | Non-heme lactoperoxidase |
|-----------------------|------------------|-------------------------|
| Milton Whey | colony forming units/ml | mg/liter whey |
| 6 h at 4 °C | <10⁹ | 25 |
| 6 h at 30 °C | 7 × 10⁷ | 31 |
| 6 h at 30 °C in the presence of antibiotics | <10⁸ | 30 |

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**Fig. 2.** Sucrose gradient centrifugation of lactoperoxidase and nonheme lactoperoxidase. Lactoperoxidase (0.8 mg) and nonheme lactoperoxidase (0.7 mg) were layered on 5 to 17% sucrose gradients in 0.1 M sodium phosphate, pβ 7.2, and centrifuged at 40,000 rpm for 14 h at 2 °C in the SW 41 Rotor (Beckman). A mixture of glucose oxidase (1), immunoglobulin G (2), and horseradish peroxidase (3) were centrifuged in the same manner; these proteins were used as markers of M = 154,000, 150,000, and 40,000, respectively. Fractions of 0.35 ml were collected from the top to the bottom of the tubes. Inset, plot of molecular weight (MW) of marker proteins versus the distance of sedimentation.
identified as glycoproteins (Fig. 4). Each protein gave a single band on SDS-polyacrylamide gel electrophoresis after staining with the Schiff’s reagent (Fig. 4). The intensity of staining for carbohydrates was similar for the two proteins (analysis at two protein inputs).

Peptide maps of lactoperoxidase and non-heme lactoperoxidase after limited proteolysis by subtilisin or trypsin are shown in Fig. 5. Subtilisin treatment (Fig. 5A) yielded two major peptides (14,000 and >8,000) which were common to the two protein forms. Action of trypsin on lactoperoxidase and non-heme lactoperoxidase gave very similar cleavage products, a major peptide of $M_\text{r} = 17,000$ and several other smaller peptides.

The relationship between lactoperoxidase and non-heme lactoperoxidase was further analyzed by an immunological approach. We checked whether non-heme lactoperoxidase was able to react with anti-lactoperoxidase antibodies. Two immune sera produced against bovine lactoperoxidase ($A_{412}/A_{280} \approx 0.8$) exhibited a high anti-lactoperoxidase antibody titer; significant binding activity for $^{125}\text{I}$-labeled lactoperoxidase could be detected at a dilution higher than 1 to $10^6$. The cross-immunological properties between heme and non-heme forms of lactoperoxidase were assessed by double immunodiffusion in agar and competitive binding experiments in liquid phase radioimmunoassay using either $^{125}\text{I}$-labeled lactoperoxidase or $^{125}\text{I}$-labeled non-heme lactoperoxidase as tracer antigen. Fig. 6A shows that non-heme lactoperoxidase ($\text{wells 2 and 5}$) as well as bovine lactoperoxidase (our preparation, $\text{wells 1 and 4}$) or a commercial preparation from Boehringer Mannheim ($\text{wells 3 and 6}$) gave a single precipitating line with an undiluted anti-lactoperoxidase antiserum. Radioimmunoassay data are reported in Fig. 6B. Both $^{125}\text{I}$-labeled lactoperoxidase and $^{125}\text{I}$-labeled non-heme lactoperoxidase bound to anti-lactoperoxidase antibodies. At a given dilution of the antiserum (1:6000 final dilution), anti-lactoperoxidase antibodies bound $^{125}\text{I}$-labeled lactoperoxidase more effectively than $^{125}\text{I}$-labeled non-heme lactoperoxidase. The antigen-antibody reactions were specific since the binding of the $^{125}\text{I}$-labeled protein was displaced by the unlabeled protein. Surprisingly, different displacement curves were obtained with the two unlabeled proteins. The binding of $^{125}\text{I}$-labeled lactoperoxidase was more readily displaceable by lactoperoxidase than by non-heme lactoperoxidase. Conversely, displacement of the binding of $^{125}\text{I}$-labeled non-heme lactoperoxidase to anti-lactoperoxidase antibodies was obtained at low concentrations of non-heme lactoperoxidase as compared to lactoperoxidase. The concentrations of unlabeled protein required to displace the binding of the corresponding $^{125}\text{I}$-labeled protein by 50% were similar, about 60 ng/ml. Similarly, the concentrations of unlabeled protein required to displace by 50% the binding of the other $^{125}\text{I}$-labeled protein were in the same range, 30–40 μg/ml. Results obtained with the two immunological approaches are in agreement with the presence of common epitopes on lactoperoxidase and non-heme lactoperoxidase.

**Binding Properties of Lactoperoxidase and Non-heme Lactoperoxidase**

**Diiodotyrosine**—Chemical iodination (with $^{125}\text{I}$) of monoiodotyrosine by chloramine-T yielded $^{125}\text{I}$-diiodotyrosine which co-chromatographed with authentic diiodotyrosine. Some minor labeled compounds were found in the labeling solution. They represented about 5–7% of total $^{125}\text{I}$. The association between $[^{125}\text{I}]$diiodotyrosine and lactoperoxidase or non-heme lactoperoxidase was measured after a 5-min incubation at
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non-heme lactoperoxidase did not interact with \[^{125}\text{I}\]diiodotyrosine whatever the amount of protein. That the interaction between lactoperoxidase and \[^{125}\text{I}\]diiodotyrosine is specific is shown on Fig. 7B. Binding of \[^{125}\text{I}\]diiodotyrosine to lactoperoxidase was progressively decreased by increasing the concentration of unlabeled diiodotyrosine in the incubation mixture. A 50% displacement of lactoperoxidase-bound \[^{125}\text{I}\]diiodotyrosine was obtained using 0.3 × 10^{-7} M diiodotyrosine.

**Tubulin**—Lactoperoxidase and non-heme lactoperoxidase coupled to Affi-Gel 10 were tested for their ability to extract tubulin from crude rat brain 100,000 × g supernatant. Protein material retained on affinity columns was eluted by 1 M NaCl and analyzed by SDS-polyacrylamide gel electrophoresis. The immobilized proteins possessed the capacity to bind tubulin (Fig. 8). Furthermore, the capacity of insolubilized lactoperoxidase and non-heme lactoperoxidase to bind tubulin was similar since an equal amount of tubulin was extracted when a given volume of rat brain supernatant was applied to the same amount of immobilized protein. The binding capacity of the two forms of lactoperoxidase was about 0.06 mg of tubulin/mg of immobilized protein, a value very similar to that obtained previously with lactoperoxidase (11). In addition to the α and β tubulin subunits, some other proteins were retained on and eluted from the affinity columns. The main contaminant had a relative molecular mass slightly lower than that of β tubulin.

**Is Non-heme Lactoperoxidase Present in the Milk of Various Origins?**

In addition to bovine milk, goat, sheep, and human milk contain both lactoperoxidase and the non-heme protein. Lactoperoxidase and the non-heme form in the various species had the same apparent molecular weight in SDS-polyacrylamide gel electrophoresis (result not shown).

**DISCUSSION**

Lactoperoxidase has been extensively used in biochemistry as a means to radioiodinate proteins and as a model of thyroid peroxidase, which was obtained in a purified state much later. In the present study, lactoperoxidase has been extracted from whey by a method previously described (3–5). The purity was higher than 90% as judged by SDS-polyacrylamide gel electrophoresis and the A_{280}/A_{410} ratio. Our preparations of lactoperoxidase were comparable to the available commercial preparations in terms of these criteria and the enzyme activity. Under the conditions used to obtain native lactoperoxidase, we have identified a protein which exhibits (a) some properties of the polypeptide chain of lactoperoxidase (basic properties, molecular weight, behavior on limited proteolysis), (b) a carbohydrate content similar to that of lactoperoxidase, (c) immunological cross-reactivity, but (d) none of the properties related to the presence of heme (absorption in the 390- to 430-nm region and enzyme activity).

It has not been determined to what extent the non-heme protein approximates structurally lactoperoxidase apoprotein. Non-heme lactoperoxidase might indeed derive from the intact form either by cleavage of the ester bond linking the heme moiety to the peptide chain or by removal of a portion of the NH₂-terminal region along with the heme after action of a protease or could represent a precursor form of lactoperoxidase to which the heme has not been linked. In each case, the variation in the molecular weight would be too small to modify its relative mobility in polyacrylamide gel electrophoresis in the presence of SDS.

Non-heme lactoperoxidase is not an artefactual product resulting from the action of rennet (used to remove casein) or incubation at room temperature since it was also obtained.
Heme and Non-heme Forms of Lactoperoxidase

The presence of a non-heme form of lactoperoxidase in milk appears of general significance since non-heme lactoperoxidase was found to exist in the milk of four mammalian species, cow, sheep, goat, and human (milk taken 8-10 days after parturition).

That milk contains both lactoperoxidase and a non-heme form of lactoperoxidase was suggested 20 years ago by Allen and Morrison (15). These authors have reported that crude bovine lactoperoxidase preparations contained large hemin-free polypeptides which reacted with anti-lactoperoxidase immune serum. In their study Allen and Morrison obtained only small amounts of the non-heme form of lactoperoxidase since the elution of their ion exchange column was stopped at 0.5 M sodium acetate. We have found that the bulk of non-heme lactoperoxidase is obtained between 0.5 and 1.0 M sodium acetate. The absence of a Soret band and the fact that non-heme lactoperoxidase is of little interest to those wishing to purify an active enzyme explains why no work was done since the original observation by Allen and Morrison.

Diiodotyrosine has been shown to influence both the rate of lactoperoxidase-cysteine and lactoperoxidase-glutathione complex formation (6) and the rate of synthesis of thyroid hormones by thyroid peroxidase (16). In each case there were either spectral or kinetic arguments suggesting a binding of diiodotyrosine to the enzyme. Our observation of a binding of labeled diiodotyrosine to lactoperoxidase is, therefore, in agreement with the above-mentioned effects of this compound on both the structural and enzymatic properties of lactoperoxidase. The absence of binding to the non-heme form might be attributed either to the lack of the heme or to the lack of a receptor site on the polypeptide chain close to the heme or to a conformational modification of a diiodotyrosine-binding site on the polypeptide chain due to the removal of the heme.

The interaction of tubulin subunits with lactoperoxidase was previously demonstrated using velocity sedimentation, gel filtration, and spectral analysis (17). The interaction of tubulin with lactoperoxidase induces a red shift in the Soret spectrum produced by tubulin was identical with that produced by thiol compounds, it was suggested that tubulin could interact with the heme moiety of lactoperoxidase through free

when extraction was performed directly from milk at temperatures never greater than 4 °C. In this last case, however, there were more contaminating proteins thereby justifying the initial coagulation step. The presence of non-heme lactoperoxidase cannot be attributed to the action of microbrial agents since we found that the amount extracted from milk was not related to the level of bacterial contamination.

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sulphhydryl groups. Our results on affinity chromatography with non-heme lactoperoxidase show that this hypothesis is not sufficient to explain the binding mechanism; the interaction between tubulin and lactoperoxidase seems to be attributable at least partly, if not completely, to the protein component of lactoperoxidase. The shift in the absorption spectrum of lactoperoxidase due to tubulin might result either from a conformational modification of the protein which would in turn determine a modification in the heme region or from an additional interaction of tubulin with the heme group.

In conclusion, the similarities in physicochemical, immunological, and binding properties between lactoperoxidase and the protein called "non-heme lactoperoxidase" indicate that these two molecules are closely related, the main differences between them being the absence of heme and related properties in non-heme lactoperoxidase. The presence of this non-heme protein in milk in amounts comparable to that of lactoperoxidase could be of biological interest; it could represent either a precursor or a degradation product of the native enzyme.

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