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The Heterogeneity of Lactoperoxidase

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Lactoperoxidase from cow's milk was separated into several fractions by chromatography on Sephadex-DEAE. The heterogeneity pattern was found to be the same all through the year. All fractions were enzymically active. The main fractions (1 and 2), probably corresponding to lactoperoxidase A and B described by Polis and Schmukler, have the same extinction coefficients at 280 mμ but differ in this respect at 412 mμ. The iron-content and specific activities, based on the absorption at 280 mμ, were the same. Lactoperoxidase prepared from a single cow was also heterogeneous.

In 1943 Theorell and Åkeson [1] isolated peroxidase from cow's milk in a crystalline form for the first time. Ammonium sulfate fractionation was used to separate the lactoperoxidase from the caseinogen, and as a last step, before crystallization, their procedure included an electrophoretic separation. The final crystalline product, which was homogeneous in the moving boundary electrophoresis, had a ratio of 0.77 between the light absorption at the maximum of the Soret band (412 mμ) and at the maximum of the protein band (280 mμ). Using rennet as a casein precipitant and introducing displacement chromatography, while maintaining the ammonium sulfate fractionation of the whey, Polis and Schmukler [2] showed that lactoperoxidase from winter and autumn milk was heterogeneous. Electrophoretically it could be separated into two enzymically active peroxidase fractions. The fraction with the highest isoelectric point, lactoperoxidase A, revealed a ratio (A_{412}/A_{280}) of 0.90 as compared to 0.77 for the other fraction, lactoperoxidase B. From spring milk, however, Polis and Schmukler were not able to isolate more than one fraction, *viz.*, the 0.77 fraction. Using ion-exchange chromatography Morrison *et al.*[3] confirmed the heterogeneity of lactoperoxidase, but through modifications of their procedure Morrison and Hultquist [4] found the heterogeneity to be somewhat inconsistent in different preparations. The latter authors suggested the following explanations of these variations. Rennet, which was used to precipitate the casein, or enzymes in the udder might act proteolytically on the peroxidase molecule and thus change some of it into another fraction. The

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heterogeneity might also be due to the production of different peroxidases by genetically different animals, since pooled milk was used in the experiments.

The experiments to be reported here were started in order further to investigate the heterogeneity of lactoperoxidase and the proposed seasonal variation of this heterogeneity.

MATERIALS AND METHODS

Milk. Unpasteurized skim milk was obtained from the dairy as fresh as possible. Pooled milk was used where not otherwise stated. Milk from a single cow was purchased from a farm in a completely untreated form except for chilling immediately after the milking. Most of the fat was separated by centrifugation in a chilled serum centrifuge (3000 *g* for 30 min at +4°C) and collected as a firm floating cake which could easily be removed and discarded.

Rennet was the Sigma Chemical Co. quality.

Amberlite CG-50 was treated according to Paléus [5] before use.

Sephadex preparations were purchased from AB Pharmacia, Uppsala, Sweden. Sephadex G-200 or Sephadex-DEAE A 50 Medium quality was suspended in distilled water and the fine particles were removed by decantation. Sephadex G-200 was then equilibrated with buffer before use. Sephadex-DEAE was activated by treatment with 0.5 M hydrochloric acid followed by distilled water, 0.5 M sodium hydroxide and then washed with excess of distilled water. Finally, the resin was equilibrated with the appropriate buffer.

CM-W cellulose was prepared according to Peterson and Sober,[6] the last alcohol-drying step, however being omitted.

Column chromatography and gel filtration. The glass columns were plugged at the bottom with a piece of cotton wool and filled to 2/3 of their height with the same buffer as that used for the equilibration of the ion-exchange material or Sephadex. A slurry of carefully equilibrated material was poured into the columns. The resin was allowed to settle by its own gravity and buffer was run through the column over-night for the final equilibration. A thin sheet of porous plastic was placed on top of the column in order to avoid disturbance of the surface of the ion-exchange material of Sephadex upon application of protein or buffer solutions. The effluent was collected in fractions of equal volume in a fraction collector. The absorbancies at 280 and 412 mμ of the different fractions were determined in a Beckman DU spectrophotometer.

Peroxidase assays were performed at 22° in 0.01 M phosphate buffer, pH 7.15, containing 7×10^{-3} M guaiacol as hydrogen donor and an appropriate amount of enzyme. The reaction was started by the addition of 5μl of H_2O_2 to give a final concentration of 1.5×10^{-4} M. The change in absorbancy at 470 mμ was recorded in a Beckman DU spectrophotometer with 1 cm cells. The method gave satisfactorily good proportionality between enzyme concentration and increase in absorbancy during the initial 12 seconds.

Iron determination. Lactoperoxidase was dialyzed for 48 h against 10^{-4} M hydrochloric acid. The iron was determined according to the sulfosalicylic acid method.[7,8]

Dry weights were estimated after drying the samples to constant weight at 110°. Before the evaporation the enzyme was extensively dialyzed against 0.02 M ammonium acetate, pH 7.0.

Ultrafiltration. The enzyme solutions were concentrated by filtration through a collodion membrane under vacuum.

Chemicals. All chemicals used were of analytical grade.

Preparative operations were performed at +4° where not otherwise indicated.

EXPERIMENTS AND RESULTS

38 l of fresh skim milk was used in the preparation described in this paper. The casein was precipitated by the slow addition of 50 mg of rennet dissolved in about 10 ml of 0.1 M phosphate buffer, pH 7.0. The milk was stirred for

1 h at 37°, and the casein was removed by filtration through cheese cloth and glass wool. Through the addition of 0.5 M ammonia, pH of the whey was adjusted to 7.0, 380 g of Amberlite CG-50 was added and the whole stirred for 3 h at room temperature.[3] The resin was allowed to settle in the cold room and the supernatant whey was sucked off and discarded. The Amberlite was then repeatedly washed with cold distilled water and packed in a glass column. The washing was continued in the column until A_{280} of the effluent was below 0.030. The proteins adsorbed onto the resin were eluted with 1 M K_2HPO_4. A distinct green zone thereupon migrated down the column, followed by a light red fraction. Visually, there appeared to be an inverse relationship between the amount of the green and the red fractions in different preparations. This observation is interesting, as Polis and Schmukler [2] have suggested a possible conversion of lactoperoxidase to a red protein found in milk.

The eluate containing the green zone was collected and dialyzed against distilled water over-night and then against 0.05 M phosphate buffer, pH 5.9. In order to concentrate the peroxidase, the protein was adsorbed on a CM-W cellulose column, equilibrated with the same buffer as was used for the dialysis, and the column was washed with buffer until the effluent was free from absorbancy at 280 mμ. The enzyme was eluted with 0.5 M phosphate buffer, pH 5.9, and further concentrated by ultrafiltration. In the CM-W cellulose step a lot of extraneous protein was removed. After dialysis the peroxidase was filtered through a column of Sephadex G-200 that was equilibrated with 0.1 M phosphate buffer, pH 5.9 containing 0.1 M sodium chloride (Fig. 1).

Fraction Nos. 66 to 79 were pooled, concentrated by ultrafiltration and chromatographed on CM-W cellulose which was in equilibrium with the same buffer as was used in the gel-filtration step. Elution with this buffer separated a small uncoloured protein impurity in front of the peroxidase fraction (Fig. 2). The peroxidase peak was quite steep in front, but showed a considerable tailing which gave a dilute eluate at the end of the peak. Other

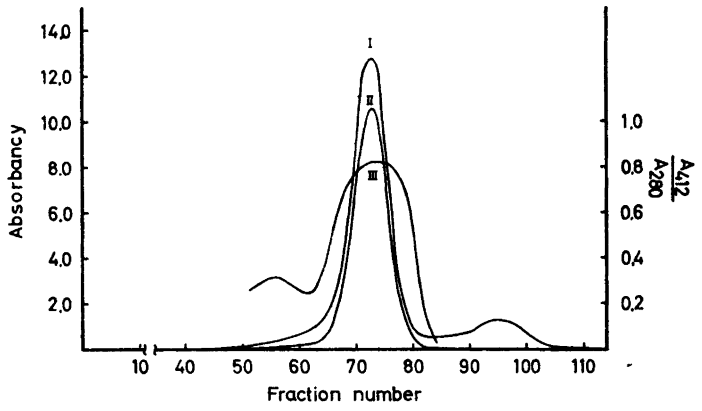

Fig. 1. Gel-filtration of lactoperoxidase on Sephadex G-200 column, 460 × 40 mm. Buffer: 0.1 M phosphate buffer, pH 5.9, containing 0.1 M NaCl. 450 mg of protein was applied. I: A_{280}; II: A_{412}; III: A_{412}/A_{280}; Fraction volume *ca.* 5 ml.

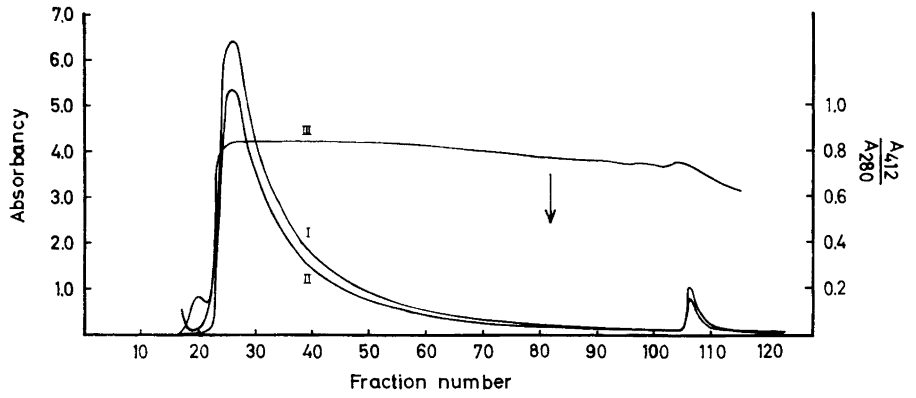

Fig. 2. Chromatography of lactoperoxidase on CM-W cellulose column, 290 × 30 mm. *Ca.* 370 mg of protein was applied. The arrow indicates change of buffer from 0.1 M phosphate buffer, pH 5.9, containing 0.1 M NaCl, to the same buffer containing 0.2 M NaCl. Fraction volume *ca.* 5 ml. For symbols see Fig. 1.

experiments have shown that it is possible to get a better separation of the first uncoloured fraction from the peroxidase fraction by lowering the ionic strength of the buffer, but an aggravated tailing of the peroxidase is then obtained. Thus, the choice of buffer is determined by striking a balance between efficient separation and an acceptable concentration of the eluted peroxidase.

Tubes Nos. 24 to 81 were pooled, and because of the large elution volume the enzyme was precipitated by the careful addition of solid ammonium sulfate to 80 % saturation. Otherwise ammonium sulfate was as far as possible avoided in the preparation procedure.

The precipitate was collected by centrifugation and dissolved in a small volume of 0.01 M Tris-HCl buffer, pH 9.0. The protein solution was extensively

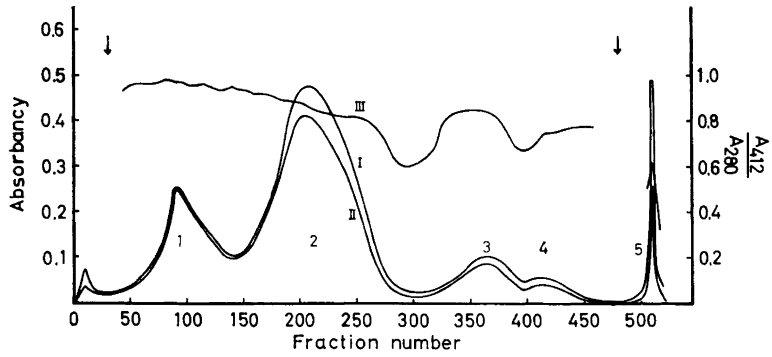

Fig. 3. Chromatography of lactoperoxidase on Sephadex-DEAE column 160 × 30 mm. 310 mg of protein was applied. The arrows indicate buffer changes (see text). Fraction volume *ca.* 5 ml. For symbols see Fig. 1 and text.

dialyzed against the same buffer, which was also used for the equilibration of the ion-exchange material, Sephadex-DEAE. Upon application of the preparation to the Sephadex-DEAE, a front fraction of lactoperoxidase migrated down the column, giving a very pronounced tailing. The main part of the peroxidase, however, was adsorbed on top of the resin. Tris-HCl buffer, pH 8.0, which was used for the continued chromatography, easily eluted the front fraction (Fraction 1, Fig. 3). Most of the protein remaining on top started to migrate, separating into three clearly visible coloured fractions (Fractions 2—4, Fig. 3). A small coloured fraction was left on the ion-exchange material and was finally eluted by 0.1 M Tris-HCl buffer, pH 7.0. Usually most of fraction 1 could be eluted from the column at pH 9.0, though it left a very slowly moving tail which was not eluted until Tris-HCl buffer, pH 8.0, was applied. Thus, fraction 2 was always somewhat contaminated by fraction 1, as is, moreover, to be seen from the decreasing ratio A_{412}/A_{280} of fraction 2. In the preparation described here fraction 1 moved so slowly that elution by Tris buffer pH 8.0 had to be started before this fraction left the column.

Fraction 1 had the highest ratio (0.99). Passing to fraction 2, the ratio gradually decreased, but after the maximum of this peak an approximately constant level around 0.82 appeared. The first two fractions are quantitatively predominant over the other three, and because of the low concentrations of the latter their ratios are rather uncertain. The peak of fraction 5 was very narrow and no constant value for the ratio was obtained. Probably this fraction is contaminated by non-specific proteins. This point was not further investigated.

All fractions (Fractions 1—5) were enzymically active, and the specific activities, based on light absorption at 280 mμ, were the same for fractions 1 and 2. Thus the specific activity based on the absorbancy at the Soret-band is highest for fraction 2.

The main peaks of fractions 1 and 2 were concentrated by ultrafiltration as soon as possible and rechromatographed on Sephadex-DEAE. Fraction 1 behaved as a homogeneous protein with the same high value of A_{412}/A_{280} as described above, whereas fraction 2 was slightly contaminated by fraction 1. This result was to be expected because of the tailing of fraction 1 in the original chromatography on Sephadex-DEAE.

It was observed that the ratio A_{412}/A_{280} for fraction 1 decreased very slowly upon standing at +4° in Tris-HCl buffer, pH 9.0. Addition of ammonium sulfate accelerated this change of the ratio A_{412}/A_{280} to a value resembling that of fraction 2. This phenomenon cannot be explained only by a lowered pH caused by the addition of ammonium sulfate, as the degree of stability was the same in 0.1 M solutions of Tris-HCl buffer, pH 9.0, phosphate buffer, pH 7.0, and acetate buffer, pH 5.0.

In order to get an idea of the reasons for the difference between fractions 1 and 2, the following spectrophotometric investigation was made. Samples from fractions 1 and 2, having absorption ratios of 0.96 and 0.85, respectively, were extensively dialyzed against a solution of 0.02 M ammonium acetate adjusted to pH 7.0 with ammonia. Light absorption spectra between 250 mμ and 560 mμ were recorded in a Beckman DK-2A spectrophotometer. The absorbancies at 280 mμ and 412 mμ were also recorded in a Beckman DU

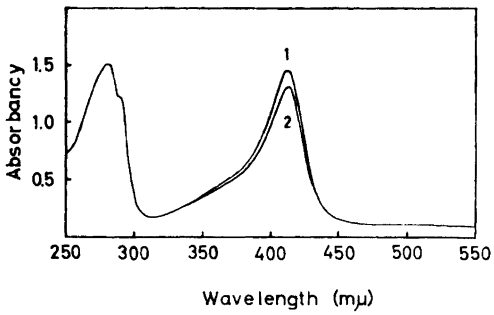

Fig. 4. Spectra of fractions 1 and 2. Concentration: 1.0 mg/ml.

spectrophotometer. Enzyme was taken for dry weight determinations and $E^{1\%}_{1\,cm}$ at 280 mμ and 412 mμ were calculated. The high ratio sample had $E^{1\%}_{1\,cm}$ = 14.8 at 280 mμ as compared to 14.9 for the sample with low ratio. At 412 mμ, however, $E^{1\%}_{1\,cm}$ was quite different for the two samples. The sample with the high ratio had an extinction coefficient of 14.2, while the sample from fraction 2 had 12.7. Light absorption spectra are shown in Fig. 4.

As the material obtained from the preparation described in this paper was not sufficient for iron determinations, these were performed on fractions 1 and 2 from other preparations. Fraction 1 yielded 0.062 % iron and fraction 2 0.061 %. The supply of enzyme was small and only two determinations for fraction 1 and three for fraction 2 were made.

Milk from a single cow was used in one preparation to find out whether the heterogeneity of lactoperoxidase could be due to genetic differences among the cows. Fresh, unpasteurized milk was treated as described in the section on materials and methods. The resin was added to the milk according to Morrison and Hultquist [4] and the preparation continued in accordance with that described above. On chromatography on Sephadex-DEAE the enzyme separated into two fractions, probably corresponding to fractions 1 and 2 as judged by the chromatographic behaviour and the light absorption ratios. Fractions 3 and 4 were not distinctly seen but the amount of enzyme applied was small and there was considerable dilution during the elution.

DISCUSSION

In this study it has been shown that lactoperoxidase can be separated into several components on Sephadex-DEAE and that the heterogeneity is not limited to two fractions, lactoperoxidase A and B, as previously described by Polis and Schmukler.[2] Judging from spectrophotometric constants and the chromatographic behaviour, the main fractions, 1 and 2, correspond to lactoperoxidase A and B, respectively. Polis and Schmukler also found that peroxidase prepared from spring milk was composed of homogeneous lactoperoxidase B in contrast to the enzyme from winter milk, which contained both lactoperoxidase A and B. In the present work it has not been possible to verify this difference between winter and spring milk. The same heterogeneity pattern

was found all through the year. The discrepancy between spring and winter milk peroxidase found by Polis and Schmukler might be explained by the fact that these authors did not use the same preparation procedure for spring and winter milk peroxidase. In preparing peroxidase from spring milk they first used the milk for the preparation of β-lactoglobulin. There is thus reason to believe that the present results are a more accurate reflection of the true nature of lactoperoxidase.

In order to find out whether the heterogeneity is due to the production of different peroxidases by genetically different cows, milk from a single cow was studied. To exclude a possible proteolytic action of rennet on lactoperoxidase, rennet was omitted from this preparation. The enzyme thus obtained consisted of at least two fractions. Neither the use of milk from one animal nor the absence of rennet changed the heterogeneity pattern. Thus, either the udder produces several peroxidases, or there is one single native peroxidase that is converted into several others during the course of the preparation. The explanation based on genetic differences among the cows is, however, not completely refuted by the present results. There is still the possibility that the production of lactoperoxidase is genetically directed in a way analogous to that for β-lactoglobulin. According to Aschaffenburg and Drewry [9] β-lactoglobulin A and B can be produced by different animals or both can be synthesized by a single cow. For lactoperoxidase this system would be more complicated, as at least four fractions of lactoperoxidase have been found. To exclude this possibility, which is less likely, a great many preparations have to be made. However, the suggestion made by Morrison *et al.* [3] that lactoperoxidase A and B represent two chemically different proteins produced by genetically different animals does not seem to be correct, as in the present study a heterogeneous enzyme was isolated from a single cow.

The light absorption at 280 mμ was found not to be responsible for the difference in A_{412}/A_{280} for fractions 1 and 2. In the ultraviolet region the spectra for these fractions were superimposable, but in the Soret-band region the absorption for fraction 2 was lower than for fraction 1. No difference in the positions of the absorption maxima was observed. Thus it seems to be no large differences between the apoproteins of the peroxidase molecules. This statement is further strengthened by the data for iron content and specific activity. A fraction with a high absorption ratio was found to contain the same amount of iron, based on dry weight, as a fraction with low ratio. The value found, however, was lower than those reported by Theorell and Pedersen [10] (0.070 %) and Polis and Schmukler [2] (0.069 %). The present value (0.062 %) is very near the 0.060 % predicted by Theorell and Pedersen [10] from their reported molecular weight of lactoperoxidase (93 000). They explained the difference between the iron-content found and that predicted as due to contamination by an iron-rich protein present in milk. Also the value found by Polis and Schmukler, however, is in good agreement with their reported molecular weight (82 000) determined by light scattering.

Spectrophotometric ratios have often been used as purity index for hemoproteins. For lactoperoxidase Allen and Morrison [11] state that the ratio A_{412}/A_{280} is directly related to the purity of the enzyme. This is probably not quite correct, as it will also depend on the relative amounts of the different fractions.

Heterogeneity is a common phenomenon among hemoproteins, and the peroxidases are not exceptional in this respect. It is difficult to suggest one reason for the heterogeneity of lactoperoxidase that is more likely than others. Perhaps the observerd conversion of fraction 1 to a protein that is spectrophotometrically similar to fraction 2 will give a suggestion of the answer.

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REFERENCES

1. Theorell, H. and Åkeson, Å. *Arkiv. Kemi, Mineral. Geol.* **17 B** (1943) No. 7.
2. Polis, B.D. and Schmukler, H.W. *J. Biol. Chem.* **201** (1953) 475.
3. Morrison, M., Hamilton, H.B. and Stotz, E. *J. Biol. Chem.* **228** (1957) 767.
4. Morrison, M. and Hultquist, D.E. *J. Biol. Chem.* **238** (1963) 2847.
5. Paléus, S. *Acta Chem. Scand.* **14** (1960) 1743.
6. Peterson, E.A. and Sober, H.A. *J. Am. Chem. Soc.* **78** (1956) 751.
7. Lorber, F. *Biochem. Z.* **181** (1927) 391.
8. Paul, K.G. *Acta Chem. Scand.* **2** (1948) 430.
9. Aschaffenburg, R. and Drewry, J. *Nature* **180** (1957) 376.
10. Theorell, H. and Pedersen, K.O. In *The Svedberg*, Almqvist & Wiksells Boktryckeri, Uppsala and Stockholm 1944, p. 523.
11. Allen, P.Z. and Morrison, M. *Arch. Biochem. Biophys.* **102** (1963) 106.

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