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Isoelectric Focusing and Separation of the Subcomponents of Lactoperoxidase

ANDERS CARLSTRÖM and OLOF VESTERBERG*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The existence of a pronounced heterogeneity of lactoperoxidase was confirmed by the method of isoelectric focusing of ampholytes. Six subfractions were demonstrated and their specific activities and spectrophotometric properties were investigated. The heterogeneity pattern obtained by isoelectric focusing was compared to the results obtained by moving boundary electrophoresis, disc electrophoresis on polyacrylamide, and sedimentation analysis. An explanation of the heterogeneity has been suggested.

The heterogeneity of peroxidase from cow's milk was first demonstrated by Polis and Shmukler.[1] Using displacement chromatography, these authors found that LPO ** from winter and autumn milk could be resolved into two fractions. Morrison *et al.*[2] confirmed the heterogeneity but later Morrison and Hultquist [3] reported the heterogeneity to be inconsistent in different preparations. They suggested that the heterogeneity was caused by the rennet used in the preparation procedure, or by genetic differences among the cows, as pooled milk was used as the source of the enzyme. However, by means of ion-exchange chromatography on Sephadex-DEAE Carlström [4] separated LPO into five fractions. No seasonal variation of the heterogeneity pattern was found and peroxidase prepared from milk from a single cow without the aid of rennet also resulted in a heterogeneous enzyme. As these results are somewhat conflicting we have further investigated the heterogeneity of LPO using another method of separation.

Isoelectric focusing of ampholytes, *e.g.* proteins, by electrolysis in natural, stable pH gradients, as described by Svensson [5] and Vesterberg and Svensson,[6] has proven valuable in the studies of the heterogeneities of myoglobin,[6,7] cytochrome c,[8] and invertase from yeast.[9] The pH gradient is obtained by electrolysis of a mixture of synthetic ampholytes of low molecular weight, carrier ampholytes. The high resolution of heterogeneous proteins is due to a

* Present address: Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden.
** Abbreviation: LPO, lactoperoxidase.

sharp focusing of the proteins at their isoelectric points; the method also permits determination of the isoelectric points by measuring the pH at the point of focusing.

MATERIALS AND METHODS

LPO from cow's milk was prepared as described earlier.[4] The chromatography on Sephadex-DEAE was, however, omitted. Enzyme concentrations were estimated spectrophotometrically using $E_{1\,cm}^{1\,\%} = 14.9$ at 280 mμ.

Crystalline rennet was a gift from Mr. B. Lindquist, Mjölkcentralen, Stockholm.

Peroxidase assays were performed in 3 ml cells at 23°C in 0.1 M phosphate buffer, pH 7.00, containing 7×10^{-3} M guaiacol as hydrogen donor and 10 μl of LPO diluted with the same buffer to give an absorbancy at 412 mμ of 0.075. The reaction was started by the addition of 10 μl of H_2O_2 to give a final concentration of 1.5×10^{-4} M. The change in absorbancy at 470 mμ in 12 sec was recorded in a Beckman DU spectrophotometer.

Carrier ampholytes were a mixture of low molecular weight aliphatic polyamino-polycarboxylic acids with different isoelectric points, synthesized and fractionated isoelectrically according to Vesterberg.[10]

Preparation of the electrolysis column and isoelectric focusing. The electrolysis column was used essentially as described by Vesterberg and Svensson.[6] The capacity of the columns used was 110 ml. The columns were siliconized with a 0.1 % (v/v) aqueous solution of Dow Corning Z-4141 in order to minimize "tailing" of the protein zones when draining the column. The density gradient was made up of sucrose (0—50 % (w/v)). The carrier ampholytes consisted of fractions isolated isoelectrically between pH 9 and 10 (0.9 g), pH 8 and 9 (0.1 g), and pH 6 and 8 (0.05 g) to make up a final concentration of approximately 1 % (w/v). 0.05 ml of concentrated phosphoric acid was added at the anode to prevent oxidation of the carrier ampholytes and 0.1 g of ethylenediamine was added at the cathode to prevent possible reduction. The columns were thermostated at + 4°C. Focusing was obtained by using a final potential of about 600 V. When the proteins were focused the columns were drained and fractions of approximately 0.8 ml were collected under a slow stream of argon to prevent absorption of CO_2.

Spectrophotometry was performed in a Beckman DU apparatus. Before determination of the ratio A_{412}/A_{280} the protein solutions were dialyzed overnight against 0.1 M phosphate buffer, pH 7.0.

Disc electrophoresis on polyacrylamide was carried out according to Ornstein and Davis [11] and Broome [12] with some modifications.[13] The polyacrylamide gels were used for electrophoresis without previous removal of the polymerizing catalysts.

Moving boundary electrophoresis was performed in a Beckman/Spinco Model H electrophoresis-diffusion instrument at + 0.8°C in an 11 ml cell. Before electrophoresis the protein solution was dialyzed against 2 l of buffer for 36 h at + 4°C.

Sedimentation analysis was made in a Beckman/Spinco Model E analytical ultracentrifuge at 59 780 rpm. The AND rotor and 12 mm cell were used.

All chemicals were of analytical grade.

EXPERIMENTS AND RESULTS

Milk from a common pool was divided into two parts. From one of these LPO was prepared with rennet included, from the other with rennet omitted from the isolation procedure. 30 mg of each preparation, having A_{412}/A_{280} ratios of 0.85 and 0.84, respectively, were applied to two columns filled with identical solutions of carrier ampholytes and sucrose. The columns were coupled in parallel circuits, which were closed for 87 h. During this time the peroxidase was focused in six sharp bands. In the column containing the enzyme prepared without rennet (expt. 3a) the fractionation pattern was indistinguishable

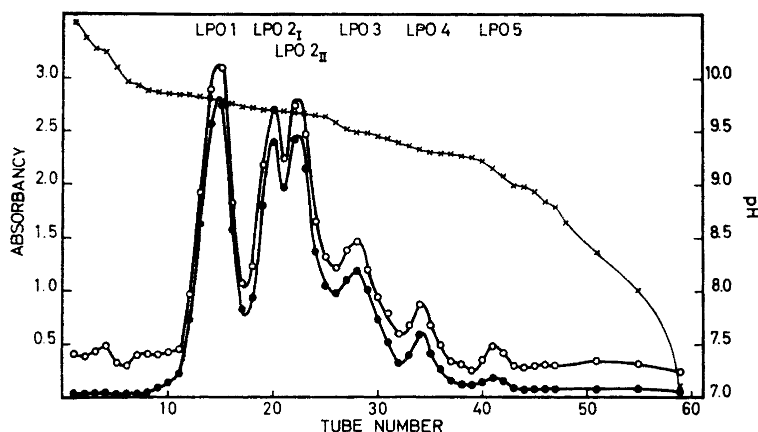

Fig. 1. Heterogeneity pattern obtained by isoelectric focusing of LPO, prepared without the aid of rennet (experiment 3a). The conditions are those described in the text. A_{412}: ●; A_{280}: ○; pH: ×.

from that seen in the column containing the peroxidase isolated using rennet (expt. 3b). The elution curve from the column containing the enzyme prepared without rennet is shown in Fig. 1. As seen from Table 1 the isoelectric points for corresponding zones in the two experiments are very similiar and within the experimental error for the pH determinations. The differences between the isoelectric points for succeeding subfractions are of about the same size (0.1—0.2 pH unit) except for that between the second and third zone, numbered from the cathode, which is only 0.01—0.03 pH unit.

To investigate the specific activities of the subfractions and to correlate the separation obtained with isoelectric focusing with that obtained by disc electrophoresis [13] an additional experiment was performed (expt. 9). Approximately 20 mg of enzyme was separated isoelectrically (Fig. 2 and Table 1).

Table 1. Isoelectric points of the subfractions of LPO determined in different experiments as described in the text.

Subfraction	Experiment 3a	Experiment 3b	Experiment 9
LPO 1	9.80	9.79	9.88
LPO 2_{I}	9.69	9.67	9.77
LPO 2_{II}	9.68	9.64	9.75
LPO 3	9.49	9.49	9.59
LPO 4	9.31	9.34	9.39
LPO 5	9.16	9.18	9.17

The column was drained, the eluent being collected in *ca.* 0.8 ml fractions. These fractions were pooled in groups (Fig. 2 and Table 2), dialyzed overnight, diluted with the appropriate buffer and subjected to activity determinations and disc electrophoresis.

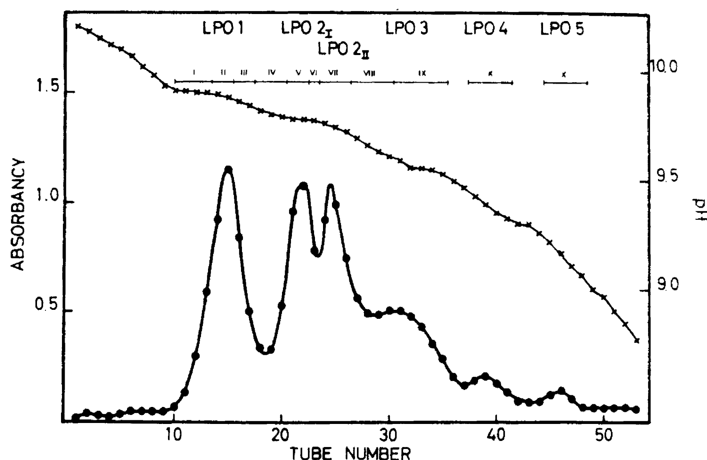

Fig. 2. Heterogeneity pattern of LPO obtained by isoelectric focusing (experiment 9). The conditions are those described in the text. Before determinations of "specific activities" and spectrophotometric ratios the tubes were pooled in groups as denoted by roman numerals and shown in Table 2. A_{412}: ●; pH: ×.

Because of the light absorption of the carrier ampholytes at 280 mμ the specific activities were based on light absorption at the Soret-band, 412 mμ. It has been observed [15] that LPO is inactivated during the course of the reaction with hydrogen peroxide and guaiacol. This inactivation is not only due to the presence of hydrogen peroxide but mainly to reaction products or intermediates in the oxidation of guaiacol, possibly free radicals. The latter fact is illustrated by a correlation between the rate of inactivation and the initial velocity of the reaction between LPO, hydrogen peroxide, and guaiacol. For this reason the enzyme concentration was adjusted to give equal

Table 2. Spectrophotometric ratios and "specific activities" of LPO fractionated in experiment 9 and as shown in Fig. 2.

Group	Tubes	A_{412}	$\frac{A_{412}}{A_{280}}$	"Spec. activity"
I	11–13	0.246	0.79	0.076 ± 0.002(n = 3)
II	14–15	0.669	0.87	0.077 ± 0.001(n = 6)
III	16–17	0.515	0.84	0.072 ± 0.002(n = 2)
IV	18–20	0.357	0.79	
V	21–22	0.693	0.84	0.069 ± 0.001(n = 6)
VI	23	0.564	0.81	
VII	24–26	0.689	0.84	0.075 ± 0.002(n = 5)
VIII	27–30	0.410	0.78	
IX	31–35	0.336	0.76	0.073 ± 0.004(n = 3)
X	38–41	0.170	0.61	0.071 ± 0.001(n = 2)
XI	45–48	0.083	0.48	0.072 ± 0.001(n = 3)

absorbancy for all subfractions at 412 mμ. The "specific activities" obtained are shown in Table 2.

The agreement between the separation obtained by disc electrophoresis and that obtained by isoelectric focusing was almost complete. Zones 2 and 3 given by isoelectric focusing could not, however, be resolved by disc electrophoresis but moved as a single band. With disc electrophoresis zone 6 was not concentrated in a sharp band like the other fractions but was located as a diffuse zone very near the origin. This latter behaviour is probably caused by ineffective "stacking" of this fraction due to its relatively low isoelectric point in comparison to the pH of the tray buffer. Analogous to previous nomenclature [13,14] the fractions are called, from cathode to anode, LPO 1, LPO 2_{I}, LPO 2_{II}, LPO 3, LPO 4, and LPO 5.

The ratios between the light absorbtion at 412 mμ and 280 mμ were also measured (Table 2). In general these ratios are lower than those found earlier [4] and there is also a marked variation in the ratios between the groups from a single peak. These results are probably due to carrier ampholytes in the enzyme solutions even after dialysis. The presence of ampholytes was demonstrated by disc electrophoresis where they were visualized by amidoschwarz as a well separated band cathodically to the LPO zones. The carrier ampholytes used have sufficiently high absorbancy in the ultraviolet region of the spectrum to explain the discrepancies with the earlier results.* This explanation is further substantiated by a correlation between the spectrophotometric ratios and the absolute amount of protein material in the tubes. In another experiment, where the carrier ampholytes were present in very low concentration after dialysis, the ratios obtained were, for LPO 1, 0.96, for LPO 2_{I} and LPO 2_{II} together, 0.90, and for LPO 3, 0.80. These results are in better agreement with the ratios found earlier.[4]

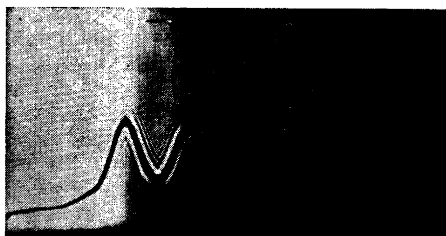

Fig. 3. Moving boundary electrophoresis of LPO, 8.5 mg/ml; phosphate buffer, $pH_{0.5^\circ} = 7.92$, $I = 0.1$, $F = 4.65$ V/cm, $t = 1093$ min. Descending boundary, cathode towards the left, A_{412}/A_{280}: 0.87 and 0.86 before and after the experiment.

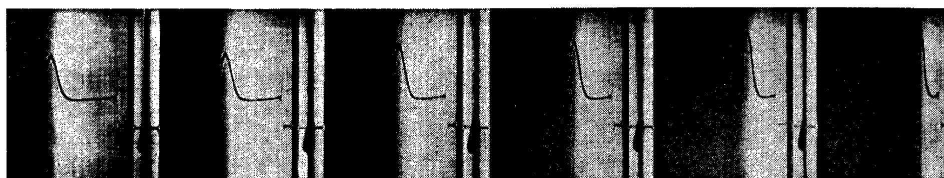

Fig. 4. Sedimentation analysis of LPO, 10.3 mg/ml in 0.05 M phosphate buffer containing 0.171 M sodium chloride, pH 7.0. Photographs were taken at intervals of 16 min. The pictures at the right represent the beginning of the run.

* Carrier ampholytes with low absorbancy in the ultraviolet region have recently been made available by LKB-Produkter AB, Stockholm, Sweden.

The high resolution of the isoelectric focusing method is demonstrated when compared to the separation of LPO obtained in the moving boundary electrophoresis (Fig. 3). The same preparation was also subjected to analytical ultracentrifugation and a symmetrical peak was demonstrated (Fig. 4). The sedimentation coefficient, calculated from this experiment, $s_{20,W} = 4.76$ S at a protein concentration of 10.3 mg/ml. Preliminary experiments indicate a significant concentration dependence of the sedimentation coefficient.

DISCUSSION

The existence of a highly heterogeneous LPO in cow's milk, earlier demonstrated by ion-exchange chromatography,[4] has thus been confirmed by the separation of this enzyme into six subfractions by the method of isoelectric focusing of ampholytes. No difference in the heterogeneity pattern was obtained if rennet was excluded from the isolation procedure of LPO. The resolving power of this method is very high and a good separation has been reported for myoglobin II_1 and II_2, which differ by 0.06 pH units in their isoelectric points.[6] By mathematical analysis, however, a resolving power of 0.02 pH units has been predicted.[6] In the experiments with LPO it has been possible to separate two subfractions, LPO 2_I and LPO 2_{II}, which show a mean difference of 0.02 pH units. As the degree of resolution is influenced by the magnitude of the diffusion coefficients of the proteins to be focused, LPO (MW 80 000—93 000) [2,4,16] would be somewhat better resolved than myoglobin (MW 18 800) [17] in this respect. The zones in the columns are much better separated than illustrated in the fractionation diagrams, as blurring of the zones is unavoidable during the draining procedure.[6]

For comparison LPO was subjected to moving boundary electrophoresis. (Fig. 3). The three major peaks correspond, with great probability, to, from cathode to anode, LPO 1, LPO 2_{I+II}, and LPO 3. Thus LPO 2_I and LPO 2_{II} are not resolved in the moving boundary electrophoresis. Otherwise the heterogeneity pattern is very similar to that obtained by isoelectric focusing except for a superior resolution of the latter method. A zone separation very much resembling that obtained by isoelectric focusing is also seen by subjecting LPO to disc electrophoresis on polyacrylamide.[13] Furthermore the correspondence between the zones obtained with these two methods was established. LPO 2_I and LPO 2_{II} could also not be resolved on polyacrylamide under the conditions for disc electrophoresis used in this investigation.

From experiments in the moving boundary electrophoresis Polis and Shmukler [1] found that the isoelectric point in phosphate buffer, $I = 0.1$, was 6.9 for LPO A and LPO B. By plotting the square root of the ionic strength against the isoelectric point, these authors obtained a value of 9.6 for the average isoionic point of LPO A. This result is in good agreement with the values for the isoelectric points presented in this paper. These latter figures are obtained in a medium of low ionic strength, which probably is free from complex-forming ions [6] and therefore should be equal to the isoionic point. However, the true isoelectric points should be some tenth of a pH unit higher, as the pH values obtained are not corrected for the effect of sucrose on the dielectric constant.[8]

The correspondence between the fractions obtained by ion-exchange chromatography and those obtained by isoelectric focusing has not yet been examined but work is now in progress. Therefore it is too early to compare the spectrophotometric ratios, A_{412}/A_{280}, and the specific activities for the subfractions derived from the two methods. However, the fractions isolated by isoelectric focusing show a decrease of the spectrophotometric ratios with decreasing isoelectric point, which is in agreement with the results obtained by ion-exchange chromatography. Also, the value $A_{412}/A_{280} = 0.99$ for the most alkaline fraction isolated by ion-exchange chromatography [4] is almost the same as the highest value found for LPO 1 by the method of isoelectric focusing (0.96). Due to absorbancy at 280 mμ by the carrier ampholytes used and the difficulty in their complete removal from the enzyme solutions the spectrophotometric ratios are lower and less reliable for the subfractions isolated by means of the latter method. This phenomenon is particularly pronounced for those fractions where the protein concentrations are low (Table 2). For this reason the estimation of the specific activities (Table 2) had to be based on the light absorption at 412 mμ. This is, however, not quite correct as the extinction coefficients at 412 mμ are probably not identical for the different subfractions.[4]

In a previous paper it was shown that LPO 1 can be converted to LPO 2 and LPO 2 to LPO 3.[14] It was suggested that the explanation for this conversion is a hydrolysis of labile amide groups of glutamine or asparagine residues in the protein. If this explanation is correct the heterogeneity pattern obtained by the method of isoelectric focusing would be consistent with two amide groups which are more labile than the others. In that case LPO 1 should correspond to the "fully amidated" molecule and by losing either one or both of the two labile groups, LPO 2_{I} or LPO 2_{II} and LPO 3, respectively, will be formed. Hydrolyzis of a third and a fourth amide residue will give rise to LPO 4 and LPO 5. If there is an appreciable difference in reactivity between the labile and less labile amide groups, which is indicated by the predominance of LPO 1 and LPO 2, other theoretical combination possibilities will probably not be detected because of low yields. The differences in isoelectric points between LPO 1, LPO 2, LPO 3, LPO 4, and LPO 5 are 0.1—0.2 pH unit and could very well reflect a difference of one unit of charge between each subfraction provided that the titration curve of the protein has a suitable derivative at the pH region of the isoelectric point, and accordingly a difference of one amide group. As LPO 2_{I} and LPO 2_{II} differ very little in isoelectric points (0.01—0.03), compared to the differences among other subfractions, it seems reasonable that the former, like isomers, have identical chemical composition, *i.e.* the number of amide groups is the same but the residue lost in LPO 2_{I} and LPO 2_{II} is different. A similiar phenomenon has been observed for cytochrome c.[8] The experiment in the analytical ultracentrifuge further supports the view that the difference between the LPO subfractions is very small, consistent with previous iron determinations.[4] However, other explanations for the heterogeneity of LPO are possible and work is continued in this laboratory on this problem.

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