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Physical and Compositional Investigations of the Subfractions of Lactoperoxidase

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In order to elucidate the chemical basis of the heterogeneity of lactoperoxidase, several subfractions were investigated for their compositions and molecular weights. From sedimentation equilibrium studies and from determinations of sedimentation coefficient, diffusion coefficient, and partial specific volume, and iron content, the molecular weight of the main component, LPO B-1, was calculated to be 78 500. The same molecular weight was also estimated for LPO B-2_I, LPO B-2_{II}, and LPO B-3. The molecular weights of the subfractions of the LPO A-group were assumed to be 76 500. Within the LPO B-group no differences were found in the amino acid and carbohydrate compositions of the subfractions investigated. The amino acid composition of the LPO A-group did not differ significantly from that of the LPO B-group. The carbohydrate content, however, was lower in the LPO A-group. Though no chemical differences were found that could explain the heterogeneity and conversions within the LPO B-group, deamidation of asparagine or/and glutamine residues might be the cause.

The molecular heterogeneity of LPO * has been thoroughly investigated in previous studies.[1,2] In accordance with the results of Polis and Shmukler,[3] LPO has been separated into two main groups, LPO A and LPO B, which were resolved further into four and six subfractions, respectively.[2] The interconversion of the four major subfractions of the LPO B-group and the conversion of these components into the corresponding subfractions of the LPO A-group was previously described and the significance of this phenomenon discussed.[2,4] Investigations of the nature of the heterogeneity of LPO in milk from individual cows show that, very probably, the enzyme is also heterogeneous in fresh milk,[2,5] and a reflection of the catabolic breakdown of LPO *in vivo*. The conversion within the LPO B-group was hypothetically attributed to consecutive deamidation reactions starting with LPO B-1.[4,1,2] The irreversible transformation between the LPO B-group and the LPO A-group

* LPO = lactoperoxidase.

has been ascribed to the release of a heterosaccharide residue,[2] as is evident from the analytical data detailed in this study, which describes, in addition, other pertinent physicochemical properties of the subfractions of LPO.

MATERIALS AND METHODS

LPO was prepared as previously described.[5] Subfractions LPO B-1, LPO B-2_I, LPO B-2_{II}, and LPO B-3 were obtained by chromatography on DEAE-Sephadex [2] and were homogeneous in disc electrophoresis, performed as described previously.[2] A preparation containing a mixture of LPO A-1 and LPO A-2_I, likewise obtained by chromatography,[2] was used as representing the LPO A-group. The enzyme samples, dissolved in 0.01 M Tris-HCl buffer, pH 9.0, were stored frozen at −15°C.

Ultracentrifugation analyses. A Beckman/Spinco Model E analytical ultracentrifuge was used. The centrifuge was equipped with a schlieren optical system, a phase plate, and a RTIC temperature unit. Before analysis, the enzyme preparations were extensively dialyzed against 1 % sodium chloride in 0.05 M phosphate buffer, pH 7.0. A 12 mm single sector cell (aluminium centerpiece) and a 12 mm double sector cell (filled Epon centerpiece) were used for the sedimentation runs at 59 780 rpm and 50 400 rpm, respectively. Sedimentation patterns of LPO B-1 in the latter cell were subjected to symmetry analyses according to Hall and Ogston.[6] Ten exposures with 8 min intervals were made in all sedimentation experiments. Data obtained from the photographic plates by means of a two-dimensional microcomparator were used for the calculations of the sedimentation coefficients according to Schachman.[7] The sedimentation coefficients, expressed in Svedberg units, S ($S = 10^{-13}$ sec), were corrected for the density and viscosity of the medium to that of pure water at 20°C as reference.[8]

Diffusion coefficients were determined from experiments performed in a double sector, synthetic boundary cell (filled Epon centerpiece), capillary type, at 5750 rpm. Calculations of diffusion coefficients were carried out using data obtained from magnifications projected onto millimeter graph paper. Areas were analyzed by mechanical planimetry. Ten exposures with 16 min intervals were made in every experiment. Calculations were performed according to the height-area method [9] using a plot in agreement with the following relationship

$$(A/H)^2 = 4\ k^2\ \pi \cdot D\ (t - t_0)$$

where A is the area between the gradient curve and the base line at time t measured from the time when the boundary was formed (t_0), H the height of that curve, k the magnification factor along the baseline, and D, the diffusion coefficient. The straight lines fitting the experimental values, were calculated by the method of least squares. The diffusion coefficients were corrected for the density and viscosity of the medium to those of water at 20°C.[8] Corrections for the dependence of the diffusion coefficients on the sedimentation coefficient were not necessary at the rotor speed used in these experiments. The results are given in Fick units, F ($F = 10^{-7}$ cm^2 sec^{-1}).

The technique of approach to sedimentation equilibrium according to Archibald as modified by Ehrenberg [10] was also employed for the molecular weight determination of LPO B-1. The runs were performed in the synthetic boundary double sector cell, capillary type, at 12 590 rpm. Distances and areas were measured from magnifications projected onto millimeter graph paper.

In all experiments performed in the ultracentrifuge, protein concentrations were determined spectrophotometrically using $E^{1\%}_{1\,cm} = 14.9$ at 280 mμ.

Partial specific volume. The density gradient technique of Hvidt *et al.*[11] was used for the determination of the partial specific volume of homogeneous LPO B-1. Before preparation of the density gradient according to Miller and Gasek,[12] Versol (obtained from AB Swedish Esso, b.p. 185−220) and bromobenzene were carefully washed with water, saturated with a 4.5 % solution of potassium bromide in water, and finally filtered through filter paper in order to remove excess water. The gradient column was immersed in a constant temperature water bath, 20±0.005°C, controlled by a Heto ultrathermostate (Heto, Birkerød, Denmark). Heating of the water bath was effected by two 150-watt lamps

connected to the thermostate. For cooling continuously running cold tap water was used. The gradient was calibrated by 1-μl drops of standard sucrose solutions [13] which were let down into the gradient. These drops were obtained from glass micropipets, the tips of which were sprayed with Teflon to give a thin hydrophobic surface layer. The positions of the drops in the gradient were estimated by means of a cathetometer, made by Ole Dich Instrumentmakers, Copenhagen, Denmark. The densities of four solutions of different LPO concentrations were subsequently determined. The enzyme solutions were beforehand equilibrated on a column of Sephadex G-25 with the same buffer as was used in the ultracentrifugation analyses. Each density value represents the mean of at least four 1-μl drops. The partial specific volume, $\bar{V}$, was calculated from the slope of a line plotted according to the following relationship.[14]

$$\bar{V} = \frac{1}{\varrho_0}\left[1-\left(\frac{\mathrm{d}\varrho}{\mathrm{d}c}\right)_{c\to 0}\right]$$

where ϱ_0 is the density at infinite protein dilution and c the concentration of protein expressed in g/ml. Protein concentrations were determined spectrophotometrically using $E^{1\%}_{1\,\mathrm{cm}} = 14.9$ at 280 mμ.

Amino acid analyses. LPO was exhaustively dialyzed first against 0.01 M Tris-HCl buffer, pH 9.0, and then dilute acetic acid, pH 4.6. To minimize destruction of amino acids,[16] hydrolysis was performed in 6 M hydrochloric acid at low protein concentrations (approximately 0.25 mg/ml). Separate samples were used for dry weight determinations. The hydrolysis tubes were immersed in a mixture of dry ice and chloroform and evacuated for 10 min to give a pressure of less than 0.005 mm Hg. The tubes were subsequently sealed and heated at 110 ± 1°C for 20, 48, 72, and 140 h, respectively. All hydrolyses were performed in duplicate. After hydrolysis, excess acid was removed in a rotary evaporator at 35–40°C. The amino acid analyses were carried out according to Moore *et al.*[17] and Spackman [18] using a Spinco Model 120 B amino acid analyzer, equipped with 0.9 × 6 cm and 0.9 × 55 cm columns. Galactosamine and glucosamine were eluted after tyrosine and phenylalanine by manual buffer change at times individually determined for each column. The buffer change affected the elution between the valine and the methionine peaks.

Cysteic acid and methionine sulphone were determined in separate samples subsequent to oxidation with performic acid according to Moore.[19] The recovery factors of this author were used.

Analysis of tryptophan was carried out according to "procedure K" of Spies and Chambers [20] after digestion with chymotrypsin and trypsin for 17 h.[21]

SH-groups were titrated in 6 M urea according to Boyer [22] and Swensson and Boyer.[23] The extinction coefficient of *p*-chloromercuribenzoate was determined by spectrophotometric titration at pH 4.5 using a solution of cysteine of known concentration.[22]

Neutral carbohydrates. Quantitative determinations were carried out by the Winzler orcinol-sulphuric acid procedure according to Francois *et al.*[24] Anhydrous orcinol was recrystallized from benzene with the aid of charcoal.[25] Mannose was used for the standard curve. The colour yield of water blanks and protein blanks (orcinol omitted) were simultaneously determined. The final results were obtained after correction for these blank values.

In addition, neutral carbohydrates were analysed after hydrolysis of LPO B-2_{II} in 0.5 M sulphuric acid at 100°C for 8 h in evacuated tubes. The protein concentration was kept low (approximately 2 mg/ml) during the hydrolysis in order to minimize interaction between carbohydrates and amino acids.[26] Excess acid and most charged molecules were subsequently removed by passing the sample through a column packed with Dowex 50-X4, 200–400 mesh (H^+-form) on top of Dowex 1-X8, 200–400 mesh (formate form).[26] The samples were then taken to dryness in a rotary evaporator and the Winzler orcinol-sulphuric acid method [24] was applied for the analysis.

Qualitative analyses of neutral monosaccharides, released and isolated as described above for the quantitative analysis, were performed by paper chromatography using Whatman No. 1 paper. The chromatographic systems used were butanol-pyridine-0.1 M hydrochloric acid (5:3:2 v/v) [28] and butanol-ethanol-water (10:1:2 v/v).[27] Aniline hydrogen phthalate was used for the detection of reducing sugars.[29]

Hexosamine analysis. Glucosamine and galactosamine were identified and quantitatively determined in the automatic amino acid analyzer. The long columns designed for analysis of acidic and neutral amino acids [18] were used; the height was reduced to 38 cm. Sodium citrate buffer, pH 4.26, 0.38 M in sodium,[17] was used for chromatographic elution. Dilute LPO samples were hydrolyzed in 4 M hydrochloric acid for 4 h at 100 ± 1°C in tubes evacuated and sealed as described for the amino acid analyses. The hydrolyzates were taken to dryness in a rotary evaporator.

Determination of sialic acid. After hydrolysis of the subfractions of LPO in 0.05 M sulphuric acid at 80°C for 1 h, sialic acid was analyzed by the thiobarbituric acid method of Warren.[30] In additional analyses, prior to quantitation, the sialic acid was separated from the remainder of the glycoprotein by passage of the hydrolyzed samples through a column of Dowex 1-X8, 50—100 mesh (formate form) according to Spiro.[26]

Iron analyses. The sulphosalicylic acid method of Lorber,[31] as modified by Paul,[32] was used. The volumes were decreased to give final volumes of 2 ml in calibrated volumetric flasks as compared to 15 ml described by Paul. The protein samples were extensively dialyzed against 0.01 M Tris-HCl buffer, pH 9.0, and dilute acetic acid, pH 4.6, in that order. Approximately 10 mg of enzyme was used for each determination. Dry weights were determined separately.

Dry weights were determined after extensive dialysis against 0.01 M Tris-HCl buffer, pH 9.0, followed by dilute acetic acid, pH 4.6. The protein samples were dried to constant weight at 105°C. Weighing was performed in a Cahn electrobalance.

Spectrophotometry was performed in a Beckman DU spectrophotometer.

The total nitrogen content was estimated by a micro-Kjeldahl procedure.

Sulphur and phosphorus analyses were made by Mikroanalyslaboratoriet, Uppsala, Sweden.

RESULTS

Sedimentation analyses. The sedimenting boundary of electrophoretically homogeneous LPO B-1 migrated as a single peak. Analysis of the Gaussian symmetry according to Hall and Ogston [6] at a protein concentration of 9.8 mg/ml showed that the protein was homogeneous in sedimentation. Sedimentation analysis of the LPO A-group, which in this study was represented by a mixture of LPO A-1 and LPO A-2_I, also showed a single symmetrical boundary as judged from the photographic plates. The same result was obtained for an unresolved preparation of the LPO B-group that, according to disc electrophoresis, contained very small amounts of the subfractions of the LPO A-group.

After concentration of LPO B-1 by ultrafiltration in 0.05 M phosphate buffer, pH 7.0, 1.0 % in sodium chloride, sedimentation revealed extensive polydispersity, although by disc electrophoresis this sample was indistinguishable from the original LPO B-1. Aggregation of LPO was never seen by concentration by ultrafiltration in 0.01 M Tris-HCl buffer, pH 9.0.

The concentration dependence of the sedimentation coefficient of LPO B-1 is demonstrated in Fig. 1. The line, calculated by means of the method of least squares, can be fitted by the equation

$$s_{20,w} = (5.19 - 0.035\ c)\ \text{S}$$

where c is the concentration of LPO B-1 in mg/ml and $s_{20,w}$ the sedimentation coefficient in pure water at 20°C. By extrapolation to infinite dilution, $s_{20,w}^{\circ}$ was found to be 5.19 S. The sedimentation coefficient of the LPO A-group was 4.80 S at a protein concentration of 9.14 mg/ml. From the equation above

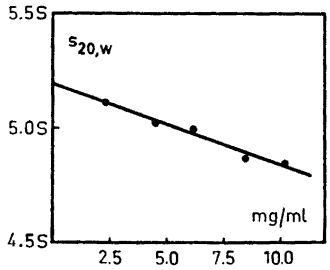

Fig. 1. Concentration dependence of the sedimentation coefficient, $s_{20,w}$, of LPO B-1. The line was fitted to the experimental points by the method of least squares.

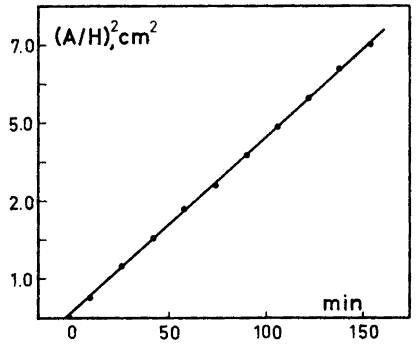

Fig. 2. A plot according to the expression of the "height-area method". Time was measured from that instance when the boundary was formed between the buffer solution, 0.05 M phosphate buffer, pH 7.0, 1.0 % in sodium chloride, and the protein solution, LPO B-1, 7.33 mg/ml, in the same buffer. From the slope of the line, which was fitted to the experimental points by the method of least squares, $D_{20,w}$ was calculated to be 6.00 F.

it is found that the sedimentation coefficient for LPO B-1 at the corresponding concentration is 4.87 S.

Diffusion coefficient. Experiments in the ultracentrifuge were performed at several concentrations of LPO B-1 as listed in Table 1. No concentration dependence of the diffusion coefficient was observed. Therefore, the mean of all determinations, 5.91 F, is taken as $D_{20,w}°$. The extrapolated t_0-values were all between 2—9 min. A representative plot of data from one experiment is shown in Fig. 2.

Partial specific volume. The concentration range employed in the determinations of the partial specific volume of LPO B-1 was 1.31—6.36 mg/ml. Three separate experiments were performed. The partial specific volumes obtained at 20°C for the protein dissolved in 0.05 M phosphate buffer, pH 7.0, and 1.0 % sodium chloride, were 0.723, 0.724, and 0.716 ml/mg, respectively. The mean

Table 1. Diffusion coefficients of LPO B-1 at different concentrations.

c mg/ml	4.34	5.98	6.30	7.33	7.54	8.31	10.3	10.3	10.6
$D_{20,w}$ F	6.01	6.02	5.72	6.00	5.79	5.96	5.91	5.77	6.01

Mean: $D_{20,w}=5.91$ F

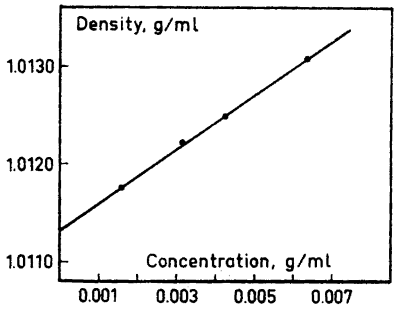

Fig. 3. A plot of density *vs.* protein concentration for LPO B-1 in 0.05 M phosphate buffer, pH 7.0, 1.0 % in sodium chloride. The slope of the line gives the buoyancy term, $(1-\overline{V}\varrho)$.

Table 2. Partial specific volume of LPO B-1. The distribution of 62 amide groups have been made according to the ratio found for aspartic acid to glutamic acid. The haem group was not included in the calculations. All hexosamine residues were assumed to be acetylated.

Amino acid or carbohydrate residue	Gram residues per 100 g of protein W	Partial specific volume $\overline{V}$	$\overline{V}W$
Aspartic acid	5.57	0.60	3.342
Asparagine	4.80	0.62	2.976
Threonine	4.07	0.70	2.849
Serine	3.62	0.63	2.281
Glutamic acid	5.26	0.66	3.472
Glutamine	4.73	0.67	3.169
Proline	5.25	0.76	3.990
Glycine	2.88	0.64	1.843
Alanine	3.39	0.74	2.509
Valine	3.60	0.86	3.096
Isoleucine	3.83	0.90	3.447
Leucine	9.82	0.90	8.838
Tyrosine	3.07	0.71	2.180
Phenylalanine	5.72	0.77	4.404
Lysine	5.51	0.82	4.518
Histidine	2.49	0.67	1.668
Arginine	7.41	0.70	5.187
Methionine	1.86	0.75	1.395
Cystine	2.04	0.61	1.244
Tryptophane	3.44	0.74	2.546
Mannose	5.37	0.61	3.276
N-Acetylglycosamine	3.65	0.67	2.446
N-Acetylgalactosamine	1.10	0.67	0.737
Total	98.48		71.413
Partial specific volume, $\overline{V}$			71.413/98.48=0.725 ml/g

value, 0.721 ml/g, was used in the calculations of the molecular weight. A representative plot of density *vs.* enzyme concentration is shown in Fig. 3.

The partial specific volume of LPO B-1 was also calculated according to Cohn and Edsall[33] from the amino acid and carbohydrate composition (*vide infra*). The amide groups were assumed to be proportionally distributed between the aspartic and glutamic acid residues. All hexosamines were considered to be acetylated. The values of the partial specific volumes used for *N*-acetyl-hexosamine and mannose were 0.67 and 0.61 ml/g, respectively.[34] The haem group was not taken into account. As seen from Table 2 the calculated partial specific volume of LPO B-1 was found to be 0.725 ml/g, in good agreement with the experimentally determined value, 0.721 ml/g.

Molecular weight. From the sedimentation coefficient, diffusion coefficient, and the experimentally determined partial specific volume, the molecular weight of LPO B-1 was calculated according to Svedbergs formula [8]

$$M = \frac{RTs}{D(1-\bar{V})}$$

and was found to be 76 400. By using the same equation and the value for s/D obtained by the technique of approach to sedimentation equilibrium, a molecular weight of 78 000 was obtained. By assuming one atom of iron per protein molecule, a molecular weight of 81 100 was calculated from the iron content of LPO B-1 (*vide infra*). The results are summarized in Table 6. The mean of these values, 78 500, was used as the molecular weight of LPO B-1 in the calculations of the amino acids and carbohydrate composition. As the ultracentrifuge experiments did not indicate heterogeneity with regard to sedimentation coefficient within the LPO B-group, the molecular weights of LPO B-2_{I}, LPO B-2_{II}, and LPO B-3 were assumed to be identical to that of LPO B-1. For reasons that will be discussed later in this paper, 76 500 was used as the molecular weight of the LPO A-group.

Molecular shape. A frictional ratio, f/f_0, equal to 1.29 was found for LPO B-1 by using the following formula [8]

$$\frac{f}{f_0} = \left[\frac{1-\bar{V}\varrho}{D^{\circ}_{20,w}{}^2 \times s^{\circ}_{20,w}}\right]^{1/3} \times 10^{-8}$$

With the assumption that this frictional ratio is due to asymmetry only, 1.29 corresponds to axial ratios of 5.6 and 6.3 for prolate and oblate ellipsoids, respectively.[8]

Amino acid and carbohydrate analysis. The recoveries of amino acids in samples of LPO B-1 hydrolyzed for 20, 48, 72, and 140 h are presented in Table 3. Serine and threonine were decomposed during the acid hydrolysis and the concentration of these amino acids in the protein was estimated by extrapolation of the recovery values to zero hydrolysis time by assuming first order kinetics for the destruction. A slight decomposition of tyrosine was also observed and the tyrosine content was obtained by a linear extrapolation to zero time.

The sum of cysteine and cystine was determined as cysteic acid, and methionine as methionine sulphone after performic acid oxidation according to

Table 3. Amino acid recoveries after acid hydrolysis of LPO B-1. The results are given in terms of μmoles/0.161 mg of protein.

Amino acid residue	Time of hydrolysis 20 h	48 h	72 h	140 h	Amino acid recoveries
Aspartic acid	0.1458	0.1461	0.1457	0.1458	0.1461[a]
Threonine	0.0627	0.0589	0.0563	0.0505	0.0648[b]
Serine	0.0614	0.0540	0.0482	0.0366	0.0668[b]
Glutamic acid	0.1245	0.1253	0.1248	0.1245	0.1253[a]
Proline	0.0866	0.0867	0.0865	0.0880	0.0870[c]
Glycine	0.0813	0.0810	0.0808	0.0808	0.0813[a]
Alanine	0.0761	0.0768	0.0764	0.0761	0.0768[a]
Valine	0.0568	0.0580	0.0584	0.0578	0.0584[a]
Methionine	0.0168	0.0164	0.0169	0.0161	0.0169[a]
Isoleucine[d]	0.0501	0.0529	0.0541	0.0545	0.0545[a]
Leucine	0.1395	0.1410	0.1394	0.1383	0.1396[c]
Tyrosine	0.0299	0.0295	0.0295	0.0289	0.0303[b]
Phenylalanine	0.0614	0.0625	0.0625	0.0619	0.0625[a]
Lysine	0.0679	0.0692	0.0688	0.0689	0.0692[a]
Histidine	0.0280	0.0292	0.0288	0.0277	0.0292[a]
Arginine	0.0756	0.0763	0.0756	0.0751	0.0763[a]
Ammonia	0.1468	0.1699	0.1762	0.1945	0.1275[b]

[a] Maximal value. [b] Extrapolated to zero hydrolysis time. [c] Mean value. [d] Includes small amounts of alloisoleucine.

The over all accuracy was ±2 % as determined from duplicate analysis for each hydrolysis time. This does not apply to the ammonia determination.

Moore.[19] As no significant amounts of methionine sulphoxide or cystine were observed, the oxidation was considered to be complete. From the analyses of samples hydrolyzed without prior oxidation, the number of methionine residues per molecule of protein was consistently found to be 8, while 11 residues were recovered in all samples after performic acid oxidation (Table 5). In spite of a lower yield in the former analyses, no methionine sulphoxide or methionine sulphone was observed, which would indicate that no partial oxidation of methionine occurred during this procedure. However, the sulphur content of LPO B-2_{II}, 1.16 %, corresponds to 28 residues of sulphur per molecule of enzyme. This result is in reasonable agreement with a composition of 11 residues of methionine and 16 residues of half-cystine. The sulphur recovery, calculated from the amino acid content of performic acid oxidized samples of LPO B-2_{II} is 1.12 %. Titration with *p*-chloromercuribenzoate did not reveal any free sulphydryl groups in LPO B-1 and LPO B-3 dissolved in 6 M urea. Therefore, all half-cystines should originate from 8 cystine residues.

Tryptophan, as analyzed by the "procedure K" of Spies and Chambers [20] after digestion with chymotrypsin and trypsin,[21] amounts to 3.44 % of LPO B-1.

The number of amide groups were estimated by an approximate extrapolation of the ammonia recoveries at different times of hydrolysis to zero time.

Table 4. Composition of LPO B-1.

Amino acid or carbohydrate residue	Grams of amino acid or carbohydrate residues per 100 g of protein[a]	Minimal molecular weight[b]	Amino acid or carbohydrate residues per 78 500 g of protein	Nearest integral No. of amino a. or carboh. residues per 78 500 g of protein	Nearest integral No. multipl. by min. mol. weight
Aspartic acid	10.45	1102	71.27	71	78 242
Threonine	4.07	2485	31.61	32	79 520
Serine	3.62	2406	32.59	33	79 398
Glutamic acid	10.05	1285	61.12	61	78 385
Proline	5.25	1850	42.44	42	77 700
Glycine	2.88	1982	39.66	40	79 280
Alanine	3.39	2097	37.46	37	77 589
Valine	3.60	2754	28.49	28	77 112
Methionine	1.38[c]	9508	8.24[c]	8[c]	76 064[c]
Isoleucine	3.83	2955	26.59	27	79 785
Leucine	9.82	1152	68.10	68	78 336
Tyrosine	3.07	5316	14.78	15	79 740
Phenylalanine	5.72	2573	30.49	30	77 190
Lysine	5.51	2326	33.76	34	79 084
Histidine	2.49	5508	14.24	14	77 112
Arginine	7.41	2108	37.22	37	77 996
Amide ammonia				62[c]	
Tryptophan [d]	3.44		14.50	15	
Total half-cystine[e]	2.04[c]		16.55[c]		
Cysteine[k]	0.00		0.00	0	
Cystine[k]	2.02		8.28	8	
Methionine[e]	1.86		11.12	11	
Mannose[f]	5.37		26.00	26	
N-Acetylglycosamine[g]	3.65		14.10	14	
N-Acetylgalactosamine[g]	1.10		4.25	4	
	98.60				
Haem	0.81[h]				
Total	99.41[i]		648.07	647	78 431

[a] Calculated from last column of Table 2. [b] Calculated from the relationship: (molecular weight of amino acid residue × 100)/percent of amino acid residue in the protein. [c] Omitted from the total. [d] Determined by the method of Spies and Chambers [20] with the modification of Harrison and Hofmann.[21] [e] Determined after performic acid oxidation according to Moore.[19] [f] Determined by the method of Winzler.[24] [g] Determined in the amino acid analyzer. All hexosamine residues were assumed to be acetylated. [h] Calculated as protohaem. [i] Total nitrogen was determined experimentally on the solution of LPO B-1 from which samples for acid hydrolyses were taken. Using the independently determined nitrogen content of 15.7 % (Table 6), a recovery in the amino acid and carbohydrate analyses of 100.5 % was calculated. [k] From titration with *p*-chloromercuribenzoate.

Table 5. Recoveries of amino acids and carbohydrate residues from LPO B-1, LPO B-2_I, LPO B-2_{II}, LPO B-3, and the LPO A-group. The molecular weights of the LPO A-group and the LPO B-group were 76 500 and 78 500, respectively. For the amino acid determinations the samples were hydrolysed for 48 h.

Amino acid or carbohydrate residue	LPO B-1	LPO B-2_I	LPO B-2_{II}	LPO B-3	LPO A
Aspartic acid	71.3	69.9	70.5	72.1	69.2
Threonine	28.7	28.3	28.6	29.5	26.0
Serine	26.3	25.8	26.0	26.8	25.8
Glutamic acid	61.1	59.6	60.9	62.3	61.8
Proline	42.3	43.3	43.2	42.3	41.4
Glycine	39.5	38.8	38.7	39.7	39.8
Alanine	37.5	36.2	37.2	37.9	37.3
Valine	28.3	27.2	27.9	28.8	28.1
Methionine[a]	8.0	8.1	8.3	7.5	8.7
Isoleucine	25.4	24.7	25.4	25.9	26.0
Leucine	68.8	66.2	66.4	68.0	66.0
Tyrosine	14.4	14.3	14.6	14.2	14.0
Phenylalanine	30.5	29.6	29.9	30.0	29.6
Lysine	33.7	32.7	31.9	32.0	34.2
Histidine	14.3	13.5	13.3	13.6	13.8
Arginine	37.2	35.9	35.7	36.5	37.0
Ammonia	82.9	79.7	80.3	81.2	77.4
Total half-cystine[b]	16.6	16.0	16.2	16.2	16.3
Methionine sulphone[b]	11.1	11.0	11.1	10.9	11.3
Mannose[c]	26.0	26.0	26.0	26.0	19.5
N-Acetylglycosamine[d]	14.1	13.9	14.0	14.4	10.5
N-Acetylgalactosamine[d]	4.3	3.8	4.5	4.3	2.8

[a] Determined without performic acid oxidation. [b] Determined according to Moore.[19] [c] See text for discussion. [d] Determined in the amino acid analyzer.

The liberation of ammonia with increasing time of hydrolysis did not follow first order kinetics. LPO B-1 was found to contain approximately 62 amide groups.

The composition of LPO B-1 is shown in Table 4.

Samples of LPO B-1, LPO B-2_I, LPO B-2_{II}, LPO B-3, and the LPO A-group were hydrolyzed for 48 h and their amino acid compositions analyzed. Cysteic acid and methionine sulphone were estimated after performic acid oxidation.[19] By using the molecular weights presented above, no significant differences in the amino acid compositions were found. The results are shown in Table 5, where also the methionine recoveries from unoxidized samples are included. Tryptophan was not analyzed except for LPO B-1. However, the light absorption at 280 mμ of the subfractions of the LPO B-group does not indicate any differences in the content of tyrosine and tryptophan.

Neutral carbohydrates in each of the subfractions LPO B-1, LPO B-2_I, LPO B-2_{II}, LPO B-3, and in the LPO A-group were quantitatively analyzed by the orcinol-sulphuric acid method of Winzler.[24] The content was found to

be 4.62 %, 4.53 %, 4.56 %, 4.63 %, and 3.35 %, respectively. However, the protein blanks, *i.e.* enzyme plus sulphuric acid, orcinol omitted, yielded rather high absorbancies. Therefore, the analysis of neutral carbohydrates was repeated for LPO B-2_{II}, but prior to the reaction with orcinol the monosaccharides were liberated by acid hydrolysis and subsequently isolated by ion-exchange chromatography. The carbohydrate content was then found to be 5.37 %. It is likely that the former lower values are explained by the protein blank absorbancies, which probably are too high. This is possibly due to chromogen formation from carbohydrate and tryptophan in the presence of sulphuric acid, but in the absence of orcinol. Therefore, as no significant differences were seen in the content of neutral carbohydrates between the subfractions of the LPO B-group, the value 5.37 % was used for all these components as seen in Table 5. The difference in the amount of neutral carbohydrates in the LPO B-group and the LPO A-group should reflect the actual difference. Thus, the content of neutral carbohydrates in the LPO A-group is taken as $5.37-(4.59-3.35)=4.13$ %.

Monosaccharides that were released from LPO B-2_{II} by acid hydrolysis and isolated by ion-exchange separation were identified by paper chromatography in two different solvent systems. In each of the solvent systems used only one spot was found that showed a positive reaction with aniline hydrogen phthalate. This reducing substance migrated with an R_F value identical to that of mannose. Mannose was also used as a standard carbohydrate in the orcinol-sulphuric acid method.

Glucosamine and galactosamine were quantitatively determined in the amino acid analyzer. The results of these analyses are summarized in Tables 5 and 6. The relatively mild hydrolytic conditions that were used for the release of the hexosamines gave rise to a considerable amount of oligo- and di-peptides. This circumstance caused a relatively large error in the estimations of the base line, and therefore the accuracy of the hexosamine determinations is smaller than that of the amino acid analyses. By increasing the hydrolysis time from 4 to 8 h there is an appreciable decrease in the concentration of peptides. However, there was no significant difference in the content of glucosamine and galactosamine as seen from the experiments with LPO B-2_I (Table 6). In our hands it was not possible to separate and analyze glucosamine and galactosamine according to Rombauts *et al.*[35]

After hydrolytic release, sialic acid was analysed by the thiobarbituric acid method of Warren.[30] LPO B-1 yielded 0.04 moles of sialic acid per mole of enzyme. However, LPO B-2_I, LPO B-2_{II}, LPO B-3, and the LPO A-group showed higher contents, 0.45, 0.18, 0.73, and 0.22 moles per mole of LPO, respectively. For these latter subfractions the absorption spectrum of the chromogen formed is the same as that formed from authentic *N*-acetylneuraminic acid, which was used as a standard in the determinations. After hydrolysis of LPO B-3, the acidic components were separated from the remainder of the glycoprotein by the ion-exchange procedure described by Spiro.[26] The acidic fraction thus obtained contained a substance that in the thiobarbituric acid method gave rise to an absorption spectrum indistinguishable from that formed from sialic acid. Unhydrolyzed samples of LPO B-3 showed a negative reaction for sialic acid.

Table 6. A comparison of physico-chemical properties of LPO.

	$E^{1\%}_{1\,cm}$ 280 mμ	$\frac{E_{412}}{E_{280}}$	N %	Fe %	Neutr. carboh. %	$GluNH_2$ [a] %	$GalNH_2$ [a] %	$s_{20,w}^{\circ}$ S	$D_{20,w}^{\circ}$ F	$\bar{V}$ ml/g	M from *S*, *D*	M from sed.eq.	M from Fe
This investigation													
LPO B-1	14.9	0.98	15.7	0.0689	5.37[b] (4.62)	3.65	1.10	5.19	5.91	0.721[c] 0.725[d]	76 400	78 000	81 100
LPO B-2_I	15.0	0.94	15.5	0.0680	5.37[b] (4.53)	3.56 3.61[g]	0.97 0.95[g]						82 100
LPO B-2_{II}	14.9	0.95	15.9	0.0697	5.37[b] (4.56)	3.62	1.16						80 100
LPO B-3	14.9	0.92	15.4	0.0709	5.37[b] (4.63)	3.73	1.12						78 800
LPO A	15.5	0.92	15.9	0.0747	4.13[b] (3.35)	2.79	0.75				76	500[b]	74 800
Theorell and Pedersen [36]	15.2	0.77		0.070				5.37[b]	5.95	0.764[c]	93 000 78 800[e]		79 800
Polis and Shmukler [3]	15.4	0.9		0.069							82	000[f]	80 900
Rombauts *et al.*[35]		0.96		0.0729	1.5	4.22	2.54			0.75[d]		77 500 69 500[e]	76 600

[a] All hexoseamines were assumed to be acetylated. [b] See text for discussion. [c] Experimentally determined. [d] Calculated. [e] Recalculated, using $\bar{V}=0.721$ ml/g. [f] From light scattering data. [g] Hydrolysed for 8 h.

Additional analyses. Extinction coefficients, nitrogen determinations, and iron analyses are summarized in Table 6. The sulphur and phosphorus contents of LPO B-2_{II} were 1.16 % and 0.006 %, respectively. These results correspond to 28 moles of sulphur and 0.2 moles of phosphorus per mole of enzyme.

DISCUSSION

The molecular weight of LPO B-1, 78 500, is the mean of the molecular weights calculated from data obtained by sedimentation and diffusion experiments, by the technique of approach to sedimentation equilibrium, and by iron analysis (Table 6). There is good agreement between this molecular weight of LPO B-1 and, (1) that obtained from the iron content of LPO as determined by Theorell and Pedersen,[36] (2) those reported by Polis and Shmukler,[3] obtained from iron analyses and from light scattering data and, (3) that which corresponds to the iron content presented by Rombauts *et al.*[35] (Table 6). However, by sedimentation and diffusion experiments, Theorell and Pedersen [36] found a molecular weight of 93 000. The partial specific volume of LPO used by these authors was 0.764 ml/g, a value which is high for a glycoprotein. Theorell and Pedersen determined the partial specific volume experimentally in a 10 ml pycnometer using a relatively low protein concentration (7.2 mg/ml). By recalculating the molecular weight, using the sedimentation and diffusion coefficients of Theorell and Pedersen but the partial specific volume determined in this study (0.721 ml/g), the molecular weight was found to be 78 800. Later Pedersen [36] reported that the molecular weight found by Theorell and Pedersen was approximately 7.5 % too high. This fact was due to unsatisfactory temperature control of the ultracentrifuge rotor. On the other hand, the sedimentation coefficient of LPO was determined at a protein concentration of 10 mg/ml. According to the relationship given by the present author for the concentration dependence of the sedimentation coefficient, the sedimentation coefficient given by Theorell and Pedersen should be 6.7 % higher at infinite dilution. Thus, this latter correction will almost completely cancel the error caused by the unsatisfactory temperature control of the rotor.

At first sight, there is very good agreement between the molecular weight obtained by Rombauts *et al.*[35] from sedimentation equilibrium studies, 77 500, with the molecular weight presently reported, 78 500. However, in their calculations these authors used 0.75 ml/g as the partial specific volume of LPO. The discrepancy between this latter value and that used in our calculations, 0.721 ml/g, corresponds to a difference of approximately 10 % in molecular weight. By using 0.721 ml/g as the partial specific volume and the s/D value of Rombauts *et al.* their molecular weight was recalculated and found to be 69 500. Therefore, there is no significance in the agreement in the molecular weight obtained from the sedimentation equilibrium data by Rombauts *et al.* and that reported in the present study. The enzyme used by Rombauts *et al.* was claimed to be homogeneous, but the relationship between their preparation and the subfractions isolated in this laboratory is not known. However, the amino acid analyses indicate a close similarity in the compositions of the enzymes used in these two studies. The discrepancy between the partial

specific volumes here reported for LPO B-1 and that calculated by Rombauts *et al.* is partly due to the lower content of neutral carbohydrates found by these latter authors. Furthermore, in their study 0.65 ml/g was used as the partial specific volume of all carbohydrates found in LPO. In our calculations, 0.61 ml/g and 0.67 ml/g were used for neutral hexoses and acetylated hexosamines, respectively.

As seen from Table 6, the results of the iron analyses of the LPO B-group are consistent with those of Theorell and Pedersen [36] and Polis and Shmukler.[3] They all correspond to molecular weights close to 80 000. The iron content of the LPO A-group and that reported by Rombauts *et al.*[35] are somewhat higher. Without doubt, the low iron content previously reported by the present author is incorrect.[5]

It is interesting to compare some physical properties of the two verdoperoxidases, LPO, and myeloperoxidase. The molecular weight of LPO is almost half of that of myeloperoxidase, 149 000.[43] LPO contains one atom of iron per molecule while myeloperoxidase contains two. The partial specific volume and frictional ratio of LPO are quite close to those of myeloperoxidase, 0.731 ml/g and 1.26, respectively.[43] A detailed comparative study of these enzymes is in progress in this laboratory.

From a previous report [2] it is known that the subfractions of the LPO A-group can be formed from the corresponding subfractions of the LPO B-group. The present sedimentation analyses showed no substantial difference between the sedimentation coefficients of the subfractions of the LPO A- and LPO B-groups. There was, however, a significant difference in the carbohydrate contents, and therefore it was thought that the difference between the LPO A- and LPO B-groups might be confined to the carbohydrate moiety. Thus comparison of the amino acid compositions of the LPO A-group and the subfractions of the LPO B-group were made by first assuming the same number of phenylalanine residues, *i.e.* 30 residues. The number of residues of the other amino acids was calculated in relation to phenylalanine and almost identical amino acid compositions were found for all the subfractions investigated. However, in relating the carbohydrate content to phenylalanine, only approximately 75 % of the carbohydrate residues found in the LPO B-group were recovered in the LPO A-group. As 78 500 was used as the molecular weight of the LPO B-group in the calculations of the amino acid and carbohydrate composition, the molecular weight of the LPO A-group, 76 500, was obtained by subtracting the sum of the molecular weights of those carbohydrate residues which constitute the difference between the LPO B- and LPO A-group, from the molecular weight of the LPO B-group.

From Table 5, which demonstrates the amino acid compositions based on the molecular weights assumed above, it appears that there are no significant differences in the amino acid compositions of the proteins analyzed. Possibly, the lower recovery of threonine in the LPO A-group has some meaning. On the other hand threonine is readily destroyed during acid hydrolysis and the extent of destruction may vary with differences in the environment of this amino acid, related perhaps to the difference in the carbohydrate contents of the LPO A-group and of the LPO B-group. If part of the carbohydrate moiety is bound by *O*-glycosidic linkages to threonine residues of

LPO, there is also a possibility that threonine is destroyed by β-elimination. If so, the β-elimination should occur concomitant to a release of carbohydrates, corresponding to the conversion of the subfractions of the LPO B-group into the subfractions of the LPO A-group. However, LPO was never exposed to the relatively extreme alkaline conditions, usually employed to cause β-elimination in glycoproteins.[38]

Analysis of LPO B-1, LPO B-2_I, LPO B-2_{II}, and LPO B-3 showed that their carbohydrate compositions are identical. The content of neutral carbohydrates reported in the present study, 5.37 %, is much higher than that found by Rombauts *et al.*,[35] 1.5 %. The lower value obtained by the latter authors is partly due to the fact that they used glucose as standard. Glucose gives a much higher molar colour yield with the anthrone reagent than does mannose.[26] In this study mannose was the only neutral carbohydrate that was found in LPO. Furthermore, Rombauts *et al.* applied the procedure used by Ota *et al.*[39] for the release of carbohydrates from the protein moiety. In this procedure the excess of hydrochloric acid was removed by evaporation to dryness. This treatment is known to cause an extensive destruction of hexoses, especially mannose.[25] Also the relatively mild conditions used for the release of mannose in this laboratory might cause a slight destruction.

The recovery of hexosamines from the subfractions of the LPO B-group, obtained by the present author, is about 70 % of that found by Rombauts *et al.*[35] in spite of the very similar hydrolytic conditions which were applied in these studies. Rombauts *et al.* separated and analyzed glucosamine and galactosamine by chromatography on the short column described by Spackman [42] for the separation of basic amino acids. In this system the hexosamines are eluted in front of tryptophan and lysine. In our hands, this method yields quite unsatisfactory separation of glucosamine and galactosamine and furthermore, the remainder of tryptophan in the hydrolysate was seriously interfering. By the procedure applied in this laboratory the yield of glucosamine and galactosamine is quite inconsistent with the results of Rombauts *et al.* (Tables 5 and 6).

Without doubt, sialic acid is not a constituent of LPO B-1. This is in agreement with the results of Rombauts *et al.*[35] However, analysis of LPO B-2_I, LPO B-2_{II}, LPO B-3, and the LPO A-group resulted in colour formation that correspond to 0.18—0.73 moles of *N*-acetylneuraminic acid per mole of protein. The content of sialic acid of LPO B-3, 0.73 moles/mole, could correspond to one residue per protein molecule. One would imagine that these results could give an indication of the reason for the heterogeneity within the LPO B-group. However, the subfractions that give positive reactions for sialic acid are the same as those which can be formed from LPO B-1, which does not contain any sialic acid. Furthermore there is no increase of colour yield that is paralleled by the change of isoelectric points of the subfractions of the LPO B-group. Therefore, it is likely that the positive colour reactions that were obtained are due to some impurity, *e.g.* unspecifically bound sialic acid.

Thus no chemical differences are found between the subfractions of the LPO B-group that could explain the heterogeneity and the conversions within this group. Deamidation of glutamine or/and asparagine residues has been

suggested as a possible cause.[4,1,2] The present investigation does not allow any conclusions regarding differences in amide groups. Analysis of amide-nitrogen according to the method of Conway [40] is not a useful method as this technique will give erratic results due to the presence of hexosamines. The method described by Marshall and Gottschalk [41] requires too much material to be applicable to this problem. Furthermore, it is doubtful whether any quantitative method presently known for analyses of amide-nitrogen could discern a difference of one amide group out of more than sixty. Further comparative investigations of the subfractions of LPO by peptide mapping are in progress, which may allow characterization of such differences.

The number of amide groups obtained in this study is an approximate figure that only provides a fair estimate of the sum of the amidated glutamatic acid and aspartic acid residues. This number was used in the theoretical calculations of the partial specific volume and by the construction of the theoretical titration curve of LPO B-1. The titration curve was drawn in order to translate the differences in isoelectric points [1] of the subfractions into differences in charge. From Fig. 4 it is seen that approximately 10 charges per mole of protein are titrated within the pH region 9.2—9.8, where the six subfractions of the LPO B-group have their isoelectric points.[1] This is about the number of charges expected if the heterogeneity within the LPO B-group is due to a consecutive deamidation. However, in the construction of the titration curve no account was taken for abnormally titrated and inaccessible groups. Furthermore, Fig. 4 shows the titration curve of the apoprotein. Therefore great care must be taken in interpreting this curve in detail. The isoelectric point of LPO B-1, as obtained from the titration curve, is 8.4, which is a

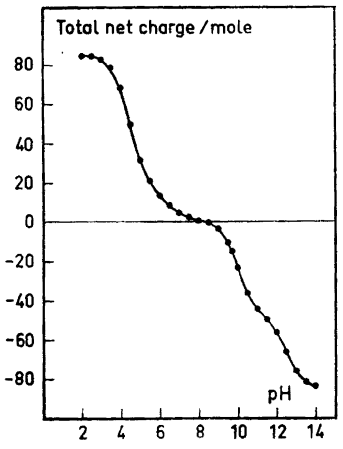

Fig. 4. A theoretical titration curve of the apoprotein of LPO B-1. Calculations were made with the assumption that there are no inaccessible or abnormally titrated groups. Terminal amino and carboxyl groups were not considered. The number of carboxyl groups was obtained by subtracting the number of amide groups from the sum of aspartic acid and glutamic acid residues found in LPO B-1. The following pK values were used.[44,45]

Ionizable group	Total number in LPO B-1	p*K* assumed
γ- and δ-Carboxyl	70	4.5
Imidazolium	14	6.5
ε-Ammonium	34	10.0
Phenolic Hydroxyl	15	10.0
Sulfhydryl	0	10.0
Guanidinium	37	12.5

low value in comparison with the experimentally determined isoelectric point, 9.8.[1] This fact would indicate that the assumed number of amide groups is a minimal value.

The carbohydrate composition of the LPO A-group, as compared to that of the LPO B-group, corresponds to a loss of 6 residues of mannose, 4 residues of glucosamine, and 1 residue of galactosamine. This difference could very well constitute one heterosaccharide residue. Thus, the conversion of each of the four major subfractions of the LPO B-group into the corresponding subfraction of the LPO A-group might be explained by the release of a heterosaccharide residue of that kind just described. This possibility has been discussed in a previous paper.[2]

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