STRUCTURE OF MILK LACTOPEROXIDASE

Evidence for a single polypeptide chain

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1. Introduction

The structure of the compound responsible for the peroxidatic activity in milk has interested many laboratories ever since the first demonstration of the activity about a century ago. The enzyme, later called lactoperoxidase [1], is a hemoprotein and its prosthetic group has recently been established to be protoheme [2]. The structure of the protein moiety has also been investigated. The M_r-value, obtained from sedimentation studies, iron content and amino acid composition, is approx. 78 000 [3,4], and it contains approx. 8–10% carbohydrates [3,4]. It has also been suggested that lactoperoxidase consists of two almost identical subunits and that the N-terminal amino acid of one chain is leucine and that of the other is blocked [3].

The number of subunits with their rel. molecular masses can be determined in SDS-PAGE, and the M_r-value of lactoperoxidase as calculated by this method is reported here. The protein has also been cleaved with CNBr at the methionine residues and the M_r-value estimated from the resulting peptide fragments. The nature and the number of the N-terminal amino acids were determined by the dansylation method. The results indicate that lactoperoxidase consists of only one polypeptide chain with leucine at the amino end.

2. Experimental

2.1. *Lactoperoxidase*

Lactoperoxidase was prepared from unpasteurized cow milk according to [5] and subsequently by the improved method of [6]. The purity index, $A_{412\ nm}/A_{280\ nm}$, of the preparation was 0.92.

2.2. *Reduction and S-alkylation of lactoperoxidase*

20 mg (approx. 250 nmol) lactoperoxidase was reduced with 50-fold molar excess of dithiothreitol (Calbiochem, A grade)/disulfide bridge (8 S–S groups/mol lactoperoxidase [4]). The reduced SH groups were alkylated with a 50-fold molar excess of iodoacetic acid (Fluka, recryst.) per SH group for 20 min in the dark [7]. The protein was separated from salts by gel filtration on a Sephadex G-25 (Pharmacia) column (1.6 × 26 cm) equilibrated with 10% acetic acid. The protein was detected by its absorbance at 280 nm and the salts by conductivity measurements.

2.3. *CNBr cleavage of lactoperoxidase*

20 mg carboxymethylated lactoperoxidase was dissolved in 1.3 ml 70% formic acid. A 100-fold molar excess of CNBr/methionine residue (11 Met/mol lactoperoxidase [4]) was added and the reaction was allowed to proceed for 24 h at 23°C [8].

2.4. *Fractionation of CNBr peptides*

CNBr peptides were fractionated by gel chromatography on a Sephadex G-50 superfine (Pharmacia) column (2.5 × 95 cm) in 10% acetic acid at a flow rate of 18 ml/h. 2.4-ml fractions were collected and measured at 280 nm.

2.5. *CNBr cleavage of cytochrome* c

6.5 mg (approx. 500 nmol) of horse cytochrome *c* (Sigma, 99%) was cleaved with a 200-fold molar excess of CNBr/methionine (2 Met/mol) as described for lactoperoxidase. The peptide fragments were not sepa-

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DNS-, 1-dimethylaminonaphthalene-5-sulfonyl; CNBr, cyanogen bromide

rated by gel chromatography, but used as a mixture as molecular mass standards in SDS-urea-PAGE.

2.6. *SDS gel electrophoresis*

SDS-PAGE of lactoperoxidase was performed at pH 7.2 in 0.1% SDS according to [9]. The ratio of crosslinker to acrylamide was 1:37. Lactoperoxidase and the M_r standards were dissolved in 10 mM sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS (recryst.) and 1% (w/v) 2-mercaptoethanol. The samples were heated for 2 min in a boiling water bath before application of approx. 5 μg of each protein/gel (6 × 80 mm). 5–15% gel concentrations were used. The electrophoresis was performed at 8 mA/gel at room temperature. Bromophenol blue was used as marker dye and the gels were stained with Coomassie Brilliant Blue [9]. The mobility of the proteins was calculated relative to the migration of the dye taking in account the length of the gels before and after staining. The M_r standards were as follows: bovine serum albumin (Sigma), M_r 68 000; chicken egg ovalbumin (grade V, Sigma), M_r 45 000; yeast cytochrome *c* peroxidase, prepared according to [10], M_r 33 400 [11]; and horse heart myoglobin (Sigma), M_r 16 950.

2.7. *SDS-urea gel electrophoresis*

CNBr peptide fractions of lactoperoxidase from gel filtration were analyzed on SDS-urea-PAGE [12]. Myoglobin, horse cytochrome *c* (M_r 12 300) and its CNBr peptides (M_r 7760, 2780 and 1810 [12]) were used as standards.

2.8. *Determination of the N-terminal amino acid*

The N-terminal amino acid of carboxymethylated lactoperoxidase was determined after reaction with DNS-Cl [13]. The DNS-amino acid was identified by high-voltage paper electrophoresis at pH 4.4 and 1.9 [13] and by thin-layer chromatography on polyamide sheets [14].

3. Results and discussion

3.1. *Determination of the* M_r*-value of lactoperoxidase*

SDS-PAGE of lactoperoxidase and the M_r standards at 10% gel concentration is shown in fig.1. Lactoperoxidase gives only one band, indicating that it consists of one peptide chain. The migration of the band is slower than that of serum albumin, showing that it has a higher M_r-value. A plot of the logarithm of M_r vs. the mobility of the protein relative to the dye, R_F, gives a M_r of 70 600 for lactoperoxidase. This value is approx. 10% lower than values obtained by amino acid analyses, from the iron content and by sedimentation experiments [3,4].

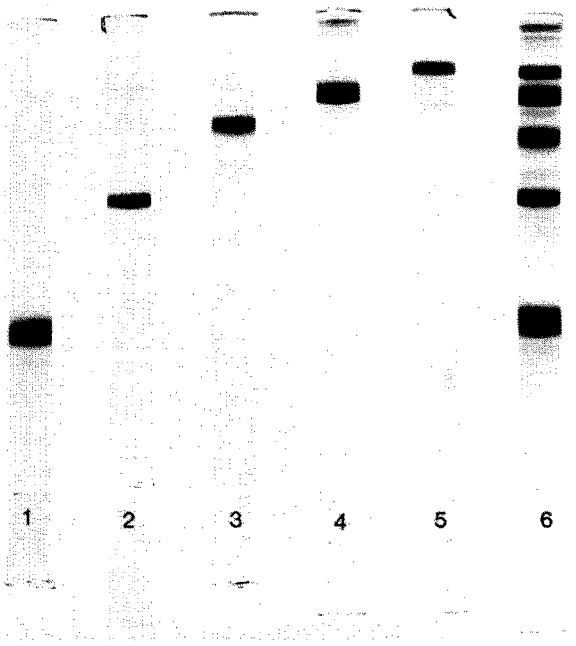

Fig.1. SDS-PAGE of lactoperoxidase at 10% gel concentration. 1, Myoglobin, M_r 16 950; 2, cytochrome *c* peroxidase, M_r 33 400; 3, ovalbumin, M_r 45 000; 4, serum albumin, M_r 68 000; 5, lactoperoxidase; 6, all proteins on the same gel.

The discrepancy between the M_r-value estimate obtained from SDS-PAGE and those from the other methods evidently depends on an anomalous migration of lactoperoxidase on SDS gels giving an incorrect M_r-value. Determination of migration rates in gels of different acrylamide concentration can be used to estimate free electrophoretic mobility (M_0) and the retardation coefficient (K_R) [15,16]. M_0 describes migration in the absence of a sieving medium and is a function of molecular charge and size. K_R is dependent solely on molecular size, and relates it to the sieving properties of the medium. Both quantities can be calculated from the Ferguson plot [17]

$$\log M = \log M_0 - K_R T$$

where M is the electrophoretic mobility in a gel of acrylamide concentration T. For convenience, relative electrophoretic mobility, R_F, is used instead of M. Plots of R_F vs. T are linear with slope K_R and intercept

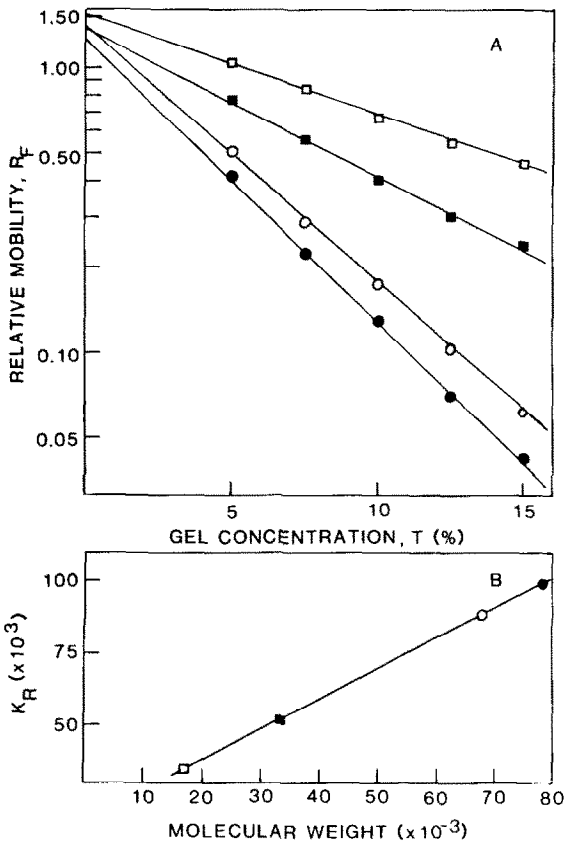

Fig.2. (A) Ferguson plot of relative mobility, R_F, vs. gel concentration T. □, myoglobin; ■, cytochrome *c* peroxidase; ○, serum albumin; ●, lactoperoxidase. (B) Secondary plot of the slope, retardation coefficient K_R, vs. the rel. molecular mass.

M_0 at $T = 0$. The M_r-value can be determined from a secondary plot of K_R against the M_r-value.

Lactoperoxidase and the standard proteins were subjected to SDS-PAGE at gel concentrations 5–15%. The Ferguson plot, R_F vs. T, is shown in fig.2A. The secondary plot, K_R vs. M_r (fig.2B), gives a M_r of 77 500 for lactoperoxidase. This value is very close to those obtained by other methods. Moreover, the result clearly establishes that lactoperoxidase consists of only one polypeptide chain.

The value for M_0, obtained from the primary plot, deviates only slightly from that of the standard proteins (fig.2A). The anomalous behaviour of lactoperoxidase (giving a 10% too low M_r estimate at 10% gel concentration) is not therefore caused by an unusual charge of the polypeptide chain, but probably by its carbohydrate content [18].

3.2. *The N-terminal amino acid of lactoperoxidase*

Determination of the N-terminal amino acid as a DNS-derivative revealed leucine. No other dansylation products than those expected, i.e. DNS-OH, DNS-NH_2, DNS-*O*-Tyr and DNS-ε-Lys, could be detected.

Quantitative determination of the N-terminal amino acid by dinitrophenyl, phenyldithiohydantion and cyanate methods have been carried out [3]. They obtained approx. 0.5 leucine/mol of lactoperoxidase, a remarkable low yield. The determination of the N-terminal DNS-amino acid with identification by electrophoresis and thin-layer chromatography is only qualitative. However, only one fluorescent spot corresponding to an α-amino acid could be obtained when using the method of [13], which is recommended for proteins. Lactoperoxidase was also carboxymethylated to guarantee a total disruption of the tertiary structure and thus to facilitate the access of the reagent to the amino terminal. The low yield of leucine obtained previously is probably a result of steric effects because the protein was not denatured by breaking the disulfide bridges.

3.3. *CNBr cleavage of lactoperoxidase*

Cleavage of lactoperoxidase with CNBr at the methionine residues resulted in 4 fractions on gel chromatography (fig.3). The first one evidently con-

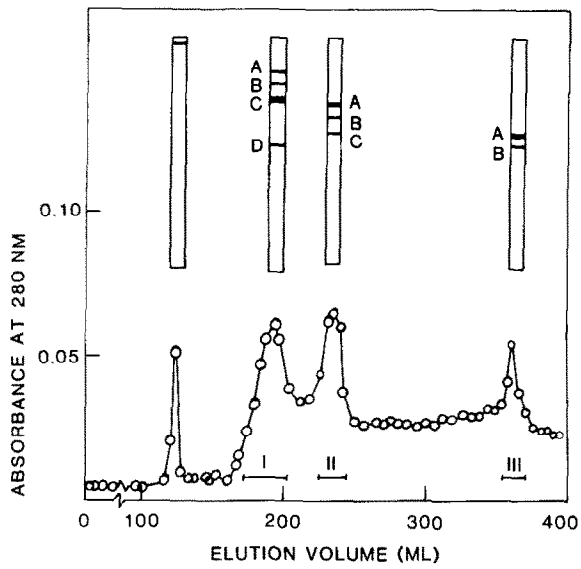

Fig.3. Gel chromatography of lactoperoxidase CNBr peptides. Sephadex G-50 superfine column (2.5 × 95 cm) in 10% acetic acid, flow rate 18 ml/h. The pooled fractions are shown with bars. Gels from SDS-urea-PAGE of the pooled fractions are shown above.

Table 1
The M_r-values of lactoperoxidase CNBr peptides, calculated from migration in SDS-urea-PAGE – nomenclature of the peptides is the same as in fig.3

CNBr peptide	M_r	CNBr peptide	M_r
IA	23 780	IIB	4420
IB	16 030	IIC	2510
IC	9380	IIIA	2570
ID	1980	IIIB	1640
IIA	6980		
		Total	69 290

tained unreacted lactoperoxidase. The pooled eluate peaks contained several peptides, which could be separated on SDS-urea-PAGE (fig.3). In all, 9 of the 12 expected peptide fragments were obtained. The M_r of these fragments were calculated from their migration on gels and are compiled in table 1. The sum of the M_r-values is approx. 69 300, 90% of the M_r obtained for lactoperoxidase. The 10% difference is explicable if a band on the gel consists of several peptides with similar M_r-values. The high recovery of the lactoperoxidase molecule from the CNBr peptides does not support the idea of two almost identical peptide chains [3]. This view was based on a peptide map of soluble tryptic peptides in which only 34 spots of the 73 expected were detected. Specific staining for histidine, tyrosine and tryptophan also gave only half of the amount expected. However, the tryptic hydrolysis was performed on protein denatured with trichloroacetic acid, in which the disulfide bridges were not broken. It is quite obvious that proteolysis cannot be complete if the peptide chain has 8 disulfide bridges keeping the separate parts of the chain together.

In conclusion, it seems clear that there is only one polypeptide chain in lactoperoxidase. The M_r-values of 77 500 obtained by SDS-PAGE is the same as previously determined by other methods. The single N-terminal amino acid, leucine, as well as the CNBr fragments support the idea of one chain.

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References

[1] Theorell, H. and Åkeson, Å, (1943) Arkiv Kemi, Mineral. Geol. 17B, No. 7.
[2] Sievers, G. (1979) Biochim. Biophys. Acta 579, 181–190.
[3] Rombauts, W. A., Schroeder, W. A. and Morrison, M. (1967) Biochemistry 6, 2965–2977.
[4] Carlström, A. (1969) Acta Chem. Scand. 13, 185–202.
[5] Carlström, A. (1965) Acta Chem. Scand. 19, 2387–2394.
[6] Paul, K. G., Ohlsson, P. I. and Henriksson, A. (1980) FEBS Lett. 110, 200–204.
[7] Waxdal, M. J., Konigsberg, W. H., Henley, W. L. and Edelman, G. M. (1968) Biochemistry 7, 1959–1966.
[8] Gross, E. (1967) Methods Enzymol. 11, 238–255.
[9] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[10] Ellfolk, N. (1967) Acta Chem. Scand. 21, 175–181.
[11] Takio, K., Titani, K., Ericsson, L. H. and Yonetani, T. (1980) Arch. Biochem. Biophys. 203, 615–629.
[12] Swank, R. T. and Munkres, K. D. (1971) Anal. Biochem. 39, 462–477.
[13] Gray, W. R. (1972) Methods Enzymol. 25, 121–138.
[14] Woods, K. R. and Wang, K.-T. (1967) Biochim. Biophys. Acta 133, 369–370.
[15] Rodbard, D. and Chrambach, A. (1970) Proc. Natl. Acad. Sci. USA 65, 970–977.
[16] Banker, G. A. and Cotman, C. W. (1972) J. Biol. Chem. 247, 5856–5861.
[17] Ferguson, K. A. (1964) Metabolism 13, 985–1002.
[18] Bretscher, M. S. (1971) Nature New Biol. 231, 229–232.