Purification, crystallization and preliminary X-ray crystallographic analysis of lactoperoxidase from buffalo milk.

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Abstract

The lactoperoxidase was prepared from buffalo milk and purified using CM-Sephadex C-50 and Sephadex G-100. The activity of the enzyme was measured using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt as a chromogenic substrate at pH 6.0. The purified protein was crystallized from 0.01 *M* sodium phosphate buffer (pH 8.0) with 10%(*v/v*) ethanol by the sitting-drop vapour-diffusion method. The green-coloured plate-like crystals are orthorhombic in space group $P2_12_12_1$ with unit-cell dimensions $a = 116.9$, $b = 103.2$ and $c = 62.3$ Å. The asymmetric unit contains one molecule with a solvent content of 52%. The crystals were stable in the X-ray beam and diffract beyond 3.2 Å. The native data to 3.5 Å have been collected and the structure determination is in progress.

Introduction

The enzyme lactoperoxidase (LPO) (E.C. 1.11.1.7) is a glycoprotein found in mammalian milk, saliva and tears (Moodbidri, Joshi & Sheth, 1976; Reiter, 1985). The enzyme catalyzes the peroxidation of endogenous thiocyanate (SCN⁻) to the antibacterial hypothiocyanite ion (OSCN⁻) (Hamon & Klebanoff, 1973) and serves as a component of the natural, non-immune biological defence system of mammals,

$$SCN^- + H_2O_2 \xrightarrow{LPO} OSCN^- + H_2O.$$

The lactoperoxidase exhibits both bactericidal and bacteriostatic effects (Reiter & Harnulv, 1984). The enzyme consists of a single polypeptide chain of 612 amino-acid residues (Cals, Mailliart, Brignon, Anglade & Dumas, 1991), a haem group, about 10% carbohydrate (Rombauts, Schroeder & Morrison, 1967), one calcium ion (Booth, Kimura, Lee Ikeda-Saito & Caughey, 1989) and a molecular mass of about 70 kDa (Sievers, 1980).

The most widely recommended industrial application of the lactoperoxidase system in food processing is in the dairy industry for the preservation of raw milk during transportation to processing plants. Further, if the lactoperoxidase system is activated immediately prior to application of approved thermal processes, the shelf-life of dairy products may be extended significantly and high-temperature processes may be replaced with more economical lower temperature treatments. In addition to energy savings, low-temperature thermal processes may provide better retention of nutrient and/or quality of food (Pruitt & Kamau, 1991). In view of such important applications of this enzyme, it is pertinent to carry out detailed structural studies. It is surprising that structural data on peroxidases from milk of any species are not available. The structures of some haem-containing peroxidases from other sources such as yeast cytochrome *c* peroxidase (Poulos *et al.*, 1980), selenoenzyme glutathione peroxidase (Epp, Ladenstein & Wendel, 1983), myeloperoxidase (Zeng & Fenna, 1992), lignin peroxidase (Edwards, Kaag, Wariishi, Gold & Poulos, 1993), recombinant *Coprinus cinereus* peroxidase (Petersen *et al.*, 1993; Petersen, Kadziola & Larsen, 1994), recombinant pea cytosolic ascorbate peroxidase (Patterson & Poulos, 1994) and manganese peroxidase (Sundaramoorthy, Kishi, Gold & Poulos, 1994) are available. The similarities and differences between lactoperoxidase and other peroxidases as well as important questions regarding specific mechanism of action have not yet been understood. We have initiated a programme of detailed three-dimensional structure determinations of lactoperoxidase from milk of various species. We report here purification, crystallization and the preliminary X-ray crystallographic investigations of buffalo lactoperoxidase.

Experimental

Purification of lactoperoxidase

The isolation and purification of lactoperoxidase was carried out at 277 K using a modification of the published procedure (Goff, Gonzalez-Vergara & Ales, 1985). 10 l of fresh raw skimmed milk of Murrah buffaloes (maintained at NDRI, Karnal) was used for lactoperoxidase preparation. The weakly acidic cation exchanger Amberlite CG 50 NH₄⁺ resin (equilibrated with 5 m*M* sodium acetate buffer, pH 6.8) was added at the rate of $22 \, g \, l^{-1}$ of the milk. The milk–resin mixture was stirred for one hour, thereafter the resin was allowed to settle for half an hour. The supernatant was decanted. The resin was washed with distilled water and sodium acetate buffer (20 m*M*, pH 6.8), until the absorbance of eluate was less than 0.02 at 280 nm. The bound protein was eluted using 500 m*M* sodium acetate buffer (pH 6.8) over a Büchner funnel. Lactoperoxidase was salted out from the above acetate buffer eluate at 70% saturation of ammonium sulfate. The enzyme was collected by centrifugation at 10 000 rev min⁻¹ for 30 min. The precipitate was taken up in 25 ml of 5 m*M* sodium phosphate buffer pH 6.8, and dialyzed overnight against the same buffer. The dialyzed lactoperoxidase solution was centrifuged at 10 000 rev min⁻¹ for 30 min to remove any particulate material and the clear greenish supernatant was collected.

The crude lactoperoxidase sample as obtained above was applied to a column of CM sephadex C-50 (Pharmacia) (3 × 10 cm) previously equilibrated with 10 m*M* sodium phosphate buffer (pH 6.8). The column was washed with 100 ml of 10 m*M* phosphate buffer (pH 6.8) containing 100 m*M* NaCl. The elution of lactoperoxidase was obtained with a linear gradient 100–200 m*M* NaCl in 10 m*M* phosphate buffer (pH 6.8). The eluate was collected from the column at the flow rate

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of 60 ml h^{-1} in 10 ml fractions. The fractions of R_z value 0.79 or higher were pooled and dialyzed against distilled water. The dialyzed lactoperoxidase sample was concentrated to about 10 ml by lyophilization and applied to the column of Sephadex G-100 (2.5 × 100 cm) previously equilibrated to 100 mM phosphate buffer (pH 6.8). The eluate was collected at a rate of 35 ml h^{-1} in 10 ml fractions. The fractions of R_z value 0.9 or higher were pooled, dialyzed against distilled water, lyophilized and stored at 253 K.

The protein concentration was determined according to the method given by Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard and absorbance at 280 nm was used to monitor protein in the column eluate (Yoshida & Xiuyun, 1991). The yield of lactoperoxidase from buffalo milk was found to be 10 mg l^{-1} which is comparable to that of 9 mg l^{-1} for bovine milk (Zhao, Fu, Wang & Wang, 1982).

The assay was carried out following the procedure of Shindler & Bardsley (1975) with some modifications. 3.0 ml of 1 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in phosphate buffer (0.1 M, pH 6.0) was mixed with 0.1 ml of sample in phosphate buffer (0.1 M, pH 7.0) containing 0.1% gelatin to initialize the spectrophotometer (Spectronic 21 D). 3.0 ml of 1 mM ABTS solution was mixed with 0.1 ml sample and 0.1 ml of 3.2 mM hydrogen peroxide solution, the absorbance was measured at 412 nm as a function of time for 2–3 min. The rate of change of absorbance was constant for at least 2 min. One unit of activity is defined as that amount of enzyme catalyzing the oxidation of 1 μmol of ABTS min^{-1} at 293 K (molar absorption coefficient 32 400 M^{-1} cm^{-1}). The peroxidase activity of buffalo milk was found to be 7.3 units ml^{-1} while that of bovine milk was 5.7 units ml^{-1}. These results indicate that buffalo milk lactoperoxidase shows higher activity than that observed for bovine milk lactoperoxidase.

The purity of lactoperoxidase was confirmed by absorbancy ratio A_{412}/A_{280} (R_z), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) and polyacrylamide gel electrophoresis (PAGE) pH 4.3 followed by staining for protein and enzyme activity using Coomassie brilliant blue R-250 and 3,3',5,5'-tetramethyl benzidine (TMBZ), respectively (Reisfeld, Lewis & Williams, 1962; Thomas, Ryan & Levin, 1976). The purified lactoperoxidase had an R_z value of 0.92 and a specific activity of 250 units mg^{-1}. The homogeneity of lactoperoxidase was observed on PAGE by staining for both protein as well as enzymatic activity. The single band on SDS–PAGE corresponded to a molecular weight of 73 kDa.

Crystallization

The purified protein was dissolved in 0.01 M sodium phosphate buffer (pH 8.0) to a final concentration ranging from 10 to 30 mg ml^{-1}. The crystallization setups were performed using the sitting-drop vapour-diffusion method (Davies & Segal, 1971) at 277 K with 0.01 M sodium phosphate to which 10%(v/v) ethanol was added as precipitant in the reservoir.

Results and discussion

The green-coloured plate-like crystals grew within about 10 d. The crystals were sensitive to temperature. For X-ray diffraction work, a crystal with dimensions 0.40 × 0.30 × 0.07 mm

was mounted in a glass capillary. Because of the small size of the crystals, the crystals were investigated using EMBL synchrontron radiation and an MAR Research imaging-plate scanner at 277 K. The diffraction extended to 3.00 Å resolution. Though several crystals were used for data collection, final native data to 3.20 Å were obtained from one crystal of dimensions mentioned above. The crystals were stable in the X-ray beam. The crystal-to-detector distance was 350 mm. At a wavelength of 0.92 Å, 64 images were collected with a rotation range of 1.0° per image. An examination of images containing zonal regions indicated that the crystal system was orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 116.9$, $b = 103.2$, $c = 62.3$ Å and $V = 7.51 \times 10^5$ Å^3. Assuming one molecule of approximate molecular weight of around 73 000 Da the value of V_m is found to be 2.57 Å^3 Da^{-1}, which is within the range of values obtained for other protein crystals (Matthews, 1968). This implies a solvent content of approximately 52%. The intensity data were integrated using the programs *DENZO* (Otwinowski, 1991) and *MOSFLM* (Leslie, Brick & Wonacott, 1986). The completeness of native data is >80% to 3.5 Å resolution and the $R_{sym(I)}$ is 9.5%, and the overall mean ratio $I/\sigma(I) = 9.5$ for the 3.5 Å resolution data set. R_{sym} is defined as,

$$\sum_{hkl}\sum_i^N |\bar{I} - I_i| / \sum_{hkl} N\bar{I},$$

where $\bar{I}$ is the mean intensity of the N reflections with intensities I_i and common indices h, k, l. The structure solution is in progress.

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