Int. J. Chem. Sci. : 2 (1), 2004, 120–125

PURIFICATION OF SHEEP LACTOPEROXIDASE FROM COLOSTRUM : CHARACTERISATION AND ANTIFUNGAL STUDIES

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ABSTRACT

The lactoperoxidase was purified from colostrum of sheep by using ion exchange chromatography on CM Sephadex C–50 and gel filtration chromatography on Sephadex G–100. The percentage of enzyme recovery was 13.9%. The specific activity of sLP was measured to be 92.2 IU^{-1} protein. The molecular weight of sLP was found to be 73 KDa and monomeric by SDS–PAGE. It is a metalloprotein as evident from UV–Visible and IR spectrum studies. Purified lactoperoxidase was found to have antifungal action against fungi such as *Pencillium chryosogenum. Aspergillus flavus, Candida albicans* and *Trichoderma sp.*

Key words : Sheep lactoperoxidase, Purification, Antifungal activity.

INTRODUCTION

The enzyme lactoperoxidase (LP) (EC 1.11.1.7) is a constituent of mamalian exocrine gland secretions viz. milk, tears, saliva, etc[1–3]. LP has been recognized as effective antimicrobial agent for many years and has been used extensively as a bactericidal agent in reducing microflora in milk[4,5]. The enzyme LP together with hydrogen peroxide and thiocyanate ions comprises antibacterial system in milk[6,7]. In biological fluids, LP catalyses the peroxidation of endogenous thiocyanate ions (SCN^-) into the antibacterial hypothiocyanite ions ($OSCN^-$).

EXPERIMENTAL

Sheep colostrum was collected from Sheep Breeding Research Station, Sandhinalla, Tamil Nadu. Defatting of sheep colostrum was done by centrifuging at 10000 rpm for about 20 min. in a refrigerated centrifuge (REMI). Defatted colostrum was diluted in 1:1 ratio with 0.025 M NaCl added, 0.05 M tris HCl buffer, pH 8. The diluted colostrum was added to already swelled C.M. Sephadex C–50 and ion exchange chromatography was done by NaCl gradient elution. The resin with bound proteins and supernatant solution was loaded onto a chromatographic

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column of length 45 cm and diameter 2.2 cm and the resin was allowed to settle well in the column. The different proteins were eluted with NaCl gradient from 0.05 to 0.5 M. The elute was collecetd from the column at flow rate of 25 mL h^{-1} in 25 mL fractinos. The enzyme assay was done for peroxidase activity by 2, 2'–azinobis (3–ethylbenzthiazoline–6–sulphonic acid) [ABTS] assay method. The assay was carried out following the procedure of Shindler and Bardsley[8]. 3 mL of 0.1 mM ABTS in 0.1 M phosphate buffer pH 6 was mixed with 0.1% gelatin to initialize spectrophotometer (UV 1601 UV–visible, Shimadzu, Japan). 3 mL 0.1 mM ABTS in 0.1 M phosphate buffer pH 6 was mixed with 0.1 mL sample in 0.1 M phosphate buffer pH 7 and 0.1 mL 3.2 mm H_2O_2 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 atleast 2–3 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 absorbance coefficient 32400 M^{-1} cm^{-1}). 1 µm of ABTS gives an absorbance of 0.032400 (32400 x 10^{-6}) i.e. one unit of activity that means 0.032400 absorbance per minute is equivalent to one unit of enzyme activity. The fractions were desalted by dialysis against distilled water three times at 4°C. Sheep lactoperoxidase (sLP) got eluted at 0.1M NaCl concentration. The fraction with peroxidase activity was concentrated by polyethylene glycol (PEG)–20000.

Concentrated sample was further purified by gel filtration on Sephadex G–100 column. 4 g of Sephadex G–100 fresh powder was boiled at 90°C for 5 hrs. in 0.05M tris HCl buffer, pH 8. The activated Sephadex G–100 degassed, was loaded on to column (length 50 cm and diameter 1.1 cm). 2 mL of concentration protein solution was loaded on the top of the resin. 20 fractions of 5 mL each were collected in sterile glass bottles at a flow rate of 30 ml h^{-1}. The fractions were screened for peroxidase activity as done earlier. Fractions with peroxidase were checked by SDS–PAGE[9]. The fractions with peroxidase activity and single band on SDS–PAGE were pooled and concentrated by PEG–20000.

Electronic spectrum was recorded on UV–visible spectrophotometer (UV–1601, Shimadzu, Japan) with a data processor. The absorbance was measured between 200–450 nm using 1 cm, path length cuvette. Infrared spectrum was recorded on infrared spectophotometer–490 (Shimadzu, Japan) equipped with a data processor.

Antifungal studies

The fungal strains were procured from Sree Chithira Thirunal Institute of Medical Sciences and Technology, Thiruvananthapuram. The fungal strains used in the study were *Pencillum chrysogenum, Aspergillus niger, Aspergillus flavus, Candida albicans, Trichoderma sp., Alternaria* and *Phytophthora meadii*.

In the initial screening, antifungal activity of the sLP was examined by using Sabourad's Dextrose Agar (SDA) medium incorporated with lactoperoxidase system. Tris HCl buffer (0.05 M) containing 400 µg protein, 10 mM hydrogen peroxide and 10 mM potassium thiocyanate

was prepared and pH was adjusted to 5.5. It was then filter sterilized and added to SDA medium kept at 45°C. The medium was then allowed to solidify as slants. These slants were then spot inoculated with fungal cultures and the tubes were incubated in a humid chamber at room temperature. After 72 hrs. of incubation, the tubes were examined for the presence of fungal growth. Sensitive strains were selected and tested for minimum inhibitory concentration (MIC). The concentrations of sLP used for MIC study ranged from 50 µg/mL to 500 µg/mL.

RESULTS AND DISCUSSION

The enzyme lactoperoxidase was purified from the colostrum of sheep by relatively simple procedures including ion–exchange chromatography on CM Sephadex C–50, dialysis and gel–filtration chromatography on Sephadex G–100. The different stages of purification are summarized in the Table 1. These procedures resulted in 2164–fold purification. The percentage of the enzyme recovery was 13.9 with specific activity 92.2 IU mg^{-1} protein.

Tale 1. Summary of purification of sheep lactoperoxidase

S.No.	Purification steps	Total protein (mg)	Total activity (mg^{-1})	Specific activity (IU mg^{-1})	Recovery (%)	Purification fold (Cumulative)
1.	Defat colostrum	16220.60	691	0.0426	100	1
2.	Ion exchange chromatography	2058	632	0.307	91.5	7.20
3.	Dialysis	720	395	0.548	57.2	12.8
4.	Gel filtration I	2.11	124.9	59.2	18.1	1389.6
5.	Gel filtration II	1.043	96.2	92.2	13.9	2164.3

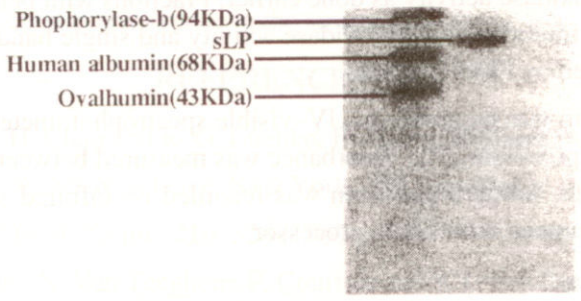

Fig. 1 SDS–PAGE showing single band SLP

The UV–visible spectrum of sLP gives absorption maxima at 280 nm and 412 nm (Fig. 2). The absorption peak at 280 nm corresponds to $\pi \rightarrow \pi^*$ transition characteristic of aromatic amino acids. The absorption peak at 412 nm attributable to $^6A_{1g} \rightarrow {}^4A_{1g}$ transition is characterstic of high spin octahedral Fe^{3+} compounds[10]. sLP is a hemeprotein.

In the IR spectrum, the peak at 3319 cm^{-1} and at 2962 cm^{-1} indicated that sLP contain an amino group and acid group. The peak at 1667 cm^{-1} shows –C=O carboxylic acid group. The

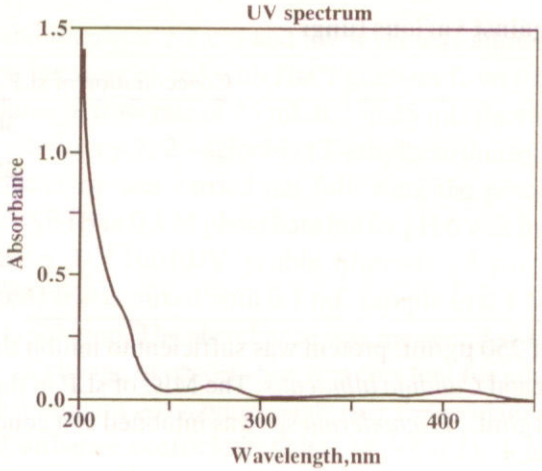

Fig. 2 UV–Visible spectrum of sLP

peak at 1546 cm^{-1} is due to N–H bending vibration (Fig. 3). Single band was obtained in SDS–PAGE that corresponds to molecular wiehgt of 73 KDa and the IR spectral studies showed the protein nature. sLP is monomeric as evident from SDS–PAGE (Fig. 1).

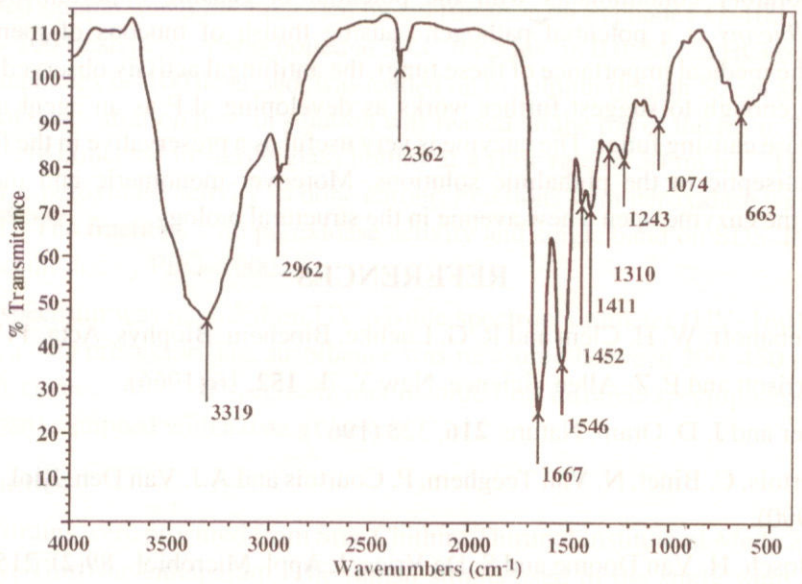

Fig. 3 IR Spectrum of sheep lactoperoxidase

In the initial screening, *Pencillium chrysogenum*, *Aspergillus niger*, *Aspergillus flavus*, *Candila albicans* and *Trichoderma sp.* were found sensitive towards sLP system. *Alternaria* and *Phytophthra meadii* were resistant. The results of minimum inhibitory concentration studies with sLP for different fungi are given in the Table 2.

Table 2. MIC of sLP against various fungi

Fungi	Concentration of sLP in the system (µg/mL)
Pencillium chryosogenum	300
Aspergillus niger	250
Aspergillus flavus	250
Candida albicans	250
Trichoderma sp.	350

The concentration of 250 µg/mL protein was sufficient to inhibit the growth of *Aspergillus niger, Aspergillus flavus* and *Candida albnicans*. The MIC of sLP in the system for *Penicillium chrysogemum* was 300 µg/mL. *Trichoderma* sp. was inhibited at a concentration of 350 µg/mL protein.

Sheep LP exhibited fungal inhibitory activity as evident from the results (Table 2). It has been reported that purified goat LP was found to have antifungal properties[11,12]. From the studies, it is learnt that a potent fungal inhibitory LP is present in the sheep colostrum.

Of the fungi tested in the present study *Pencillium chrysogenum* and *Aspergillus niger* are common laboratory contaminants with the potential of causing opportunistic infections. *Candidala albicans* is a potential pathogen causing thrush of mucous of membranes. On considering the medical importance of these fungi, the antifungal activity observed in this study is significant enough to suggest further works as developing sLP as an agent against these potential disease causing fungi. The enzyme is very useful as a preservative in the food industry and as an antiseptic in the phthalmic solutions. Moreover monomeric and metalloprotein behaviour of the enzyme open a new avenue in the structural biology.

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Accepted 12.1.2004