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Influence of pH, Temperature and Metallic Behaviour of Ovis Lactoperoxidase

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Circular dichroism (CD) spectra of sheep lactoperoxidase (sLP) were recorded in the wavelength region 190–250 nm. The spectrum shows negative CD maximum at 207 and 216 nm. It also shows that sLP maintains its structural integrity till 68°C and on further increase in temperature a sharp loss in CD spectrum was observed. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and electron paramagnetic resonance (EPR) spectrum analysis revealed that sLP contains calcium, iron and also copper.

Key Words: Ovis lactoperoxidase, pH, Temperature, Metallic behaviour.

INTRODUCTION

The enzyme lactoperoxidase (LP) (EC 1.11.1.7) is a constituent of mammalian exocrine gland secretions, *viz.*, milk, tears, saliva etc.[1]. LP is a natural bacterial defence system through the oxidation of thiocyanate ions SCN^-, by hydrogen peroxide, producing a weak oxidizing agent, hypothiocyanite ion ($OSCN^-$) with antimicrobial properties[2, 3]. Both these are present in biological fluids together with LP and are termed as lactoperoxidase system (LP-S). LP-S has proved to be both bactericidal and bacteriostatic to a wide variety of micro-organisms[4]. The enzyme consists of a single polypeptide chain of 612 amino acid residues[5, 6], a molecular mass of approximately 78000 Da[7], about 10% of which is carbohydrate[8], a covalently bound heme and one calcium ion[9].

In the present study, influence of pH and temperature on sLP was examined by using CD hoping that it would help to provide information on its stability. ICP-AES and EPR spectroscopic techniques were used to examine the metallic behaviour of sLP.

EXPERIMENTAL

LP was isolated and purified from sheep colostrum by simple procedures including ion exchange chromatography on CM Sephadex C-50 (Sigma) and gel filtration chromatography on Sephadex G-100 (Sigma). The presence of sLP containing fractions was detected by ABTS assay method[10]. The specific activity of sLP was measured to be 92.2 IU mg^{-1} at 30°C. The fractions with sLP were pooled, lyophilized and was used for spectroscopic studies. CD spectrum was

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recorded on Jasco J-715 spectropolarimeter equipped with a data processor. The scan speed was kept at 100 nm/min and response time was 4 sec. sLP concentration was 20 μM in 0.05 M Tris HCl buffer pH 8 and 1 cm path length cuvette was used. The experiments were repeated with different pH in the range of 3 to 12 CD spectrum was recorded in a thermostated cell holder (Peltier device) maintained at varying temperatures and samples were heated for 30 min at the required temperature in the same buffer before the measurements. Metal contents were determined by ICP-AES with Labtam-8410 plasma scan spectrometer which provided accurate determination of metals. EPR of sLP was recorded on EPR Varian E-112 spectroscope in powder and solution state at room temperature.

RESULTS AND DISCUSSION

Fig. 1 shows the CD spectrum of sLP recorded from 190–250 nm. There are two transitions in the far-ultraviolet CD spectrum (200–250 nm). The spectrum shows negative CD maximum at 207 nm corresponding to ($\pi \rightarrow \pi^*$) amide transition. Another CD maximum is at 216 nm corresponding to $n \rightarrow \pi^*$ amide transition and is also negative in sign. The molar ellipticities at 207 nm and at 216 nm were -23.5×10^{-4} (deg cm^2 dmol^{-1}) and -17×10^{-4} (deg cm^2 dmol^{-1}) respectively.

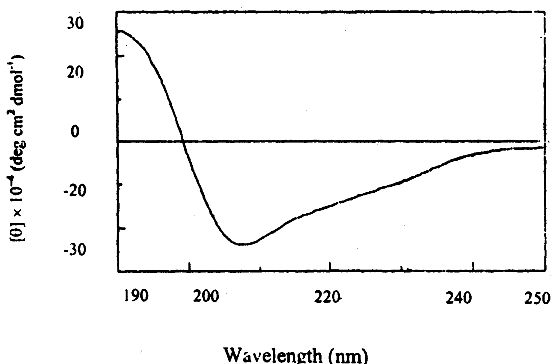

Fig. 1. Native CD spectrum of sLP in 0.05 M tris HCl buffer pH 8

The sLP was found to be stable within the pH range 5–9 (Figs. 2 and 3). The hydrogen bonding network of side chains residues is known to stabilize the α-helix and can be designed into an α-helix to enhance stability. Above pH 9, denaturation may be fast due to breaking of hydrogen bonds. The pH lower than 5 was found to be detrimental to the enzyme. The nature of buffer did not seem to influence the enzyme activity.

There is no loss of secondary structure in sLP till 68°C. On further increase in the temperature a sharp loss in CD spectrum was observed (Figure 4). The visual inspection of the sample in the cuvette suggest that thermal denaturation is accompanied by the precipitation of the enzyme and loss of CD band intensity is caused by precipitation of the protein.

ICP-AES analysis data indicate presence of 0.726 ppm calcium, 0.552 ppm iron and 0.760 ppm copper in 0.844 mg^{-1} sLP. The ratios of calcium, iron and

copper with respect to protein molecule was found to be 1.86, 1.013 and 1.225 respectively.

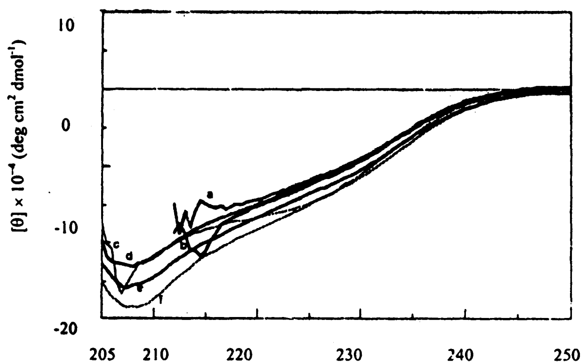

Fig. 2. CD spectra of sLP in 0.05 M tris HCl buffer: (a) pH 12 (b) pH 11 (c) pH 10 (d) pH 9, (e) pH 8 and (f) pH 7

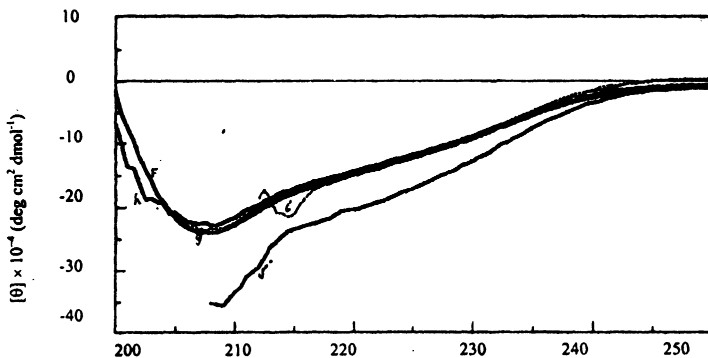

Fig. 3. CD spectra of sLP in 0.05 M tris HCl buffer: (f) pH 7 (g) pH 6 (h) pH 5 (i) pH 4 and (j) pH 3

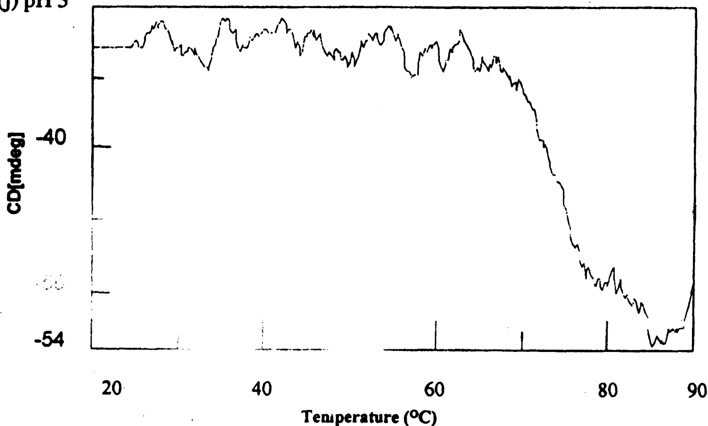

Fig.4. Thermal denaturation of sLP

Fig. 5 represents EPR spectrum of the sLP in powder state at room temperature using DPPH free radical as the 'g' marker. EPR signal for (Cu^{2+}) ions without hyperfine or superfine splitting and also without $g_{\parallel}$ and $g_{\perp}$ separation was obtained. Sheep enzyme contains copper in the unpaired electron (Cu^{2+}) state. From the graph, $g_{average}$ value was found to be 2.241 indicating copper species in the enzyme molecule. EPR spectrum showed unsplitted spectral pattern in the region expected for Fe^{3+} probably because of extremely low concentration of iron. EPR spectrum of sLP in the solution state at room temperature showed same signal as that of solid spectrum.

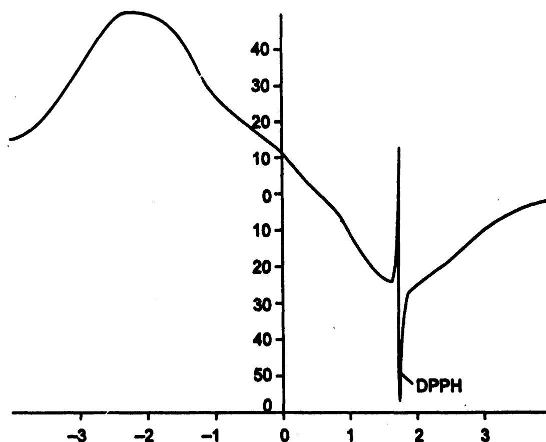

Fig. 5. Electron paramagnetic resonance spectrum of powder state of sLP at room temperature. Scah range 5000 gauss, field set 2500 gauss and microwave frequency 9.01 GHz

In view of growing applications of LP in biological and industrial fields, its pH and temperature stability is significant. A change in the stability of non-covalent bond leading to denaturation can in turn be caused by a change in pH, ionic strength and temperature. The presence of prosthetic groups, co-factors and substrates of a protein may also affect the stability of native conformation[11]. sLP was found to be stable within the pH range 5–9. The optimum pH for the enzyme activity was determined at pH 8 using 0.05 M tris HCl buffer.

Ewes' LP appears to be heat-resistant with the enzyme not being inactivated by pasteurization. Heat treatment at 63°C for 30 min left sufficient activity to catalyze the oxidation of thiocyanate[12]. The heating contributed considerable changes to the milk components; especially LP activity was lost by 13.9% at 72.5°C and 100% at 80°C during continuous flow pasteurization[13]. The present thermal stability studies of sLP show that it maintains its activity up to 68°C. On further increase in temperature it undergoes denaturation gradually. This finding agrees with previously reported results[12–16]. The secondary structure of bovine LP indicates that it contains mixture of α-helices and β-sheets[7, 17]. The CD spectrum of native sLP shows negative bands at 207 nm and 216 nm which are characteristic spectra of mixture of α and β type of proteins. This is in good agreement with the results of previous studies[7, 17]. Metallic analysis of sLP by

IGP-AES showed the presence of calcium, iron and copper. EPR signal indicated copper in the unpaired electron state in sLP molecule.

These findings lead to a conclusion that sLP contains Ca, Fe and also Cu. The metallic analysis of Ca and Fe in sLP by ICP-AES and EPR techniques agree with previous reports[9, 17, 18]. Amino acid sequence analysis of human MP[9] revealed two Ca binding sites and the present study of sLP (1.86 Ca/mol of protein) conforms to it. Similarly Cu also may have more than one binding site (1.225 Cu/mol of protein). The presence of Cu in goat milk LP has been reported which expresses more affinity towards Cu than Fe[19]. It may be assumed that the Cu binding sites of goat milk LP[19] and sLP are different. Presence of almost equal amounts of Cu and Fe shows that there is no difference in affinity towards them and they may not be sharing the same site as proposed for goat milk LP[19]. Sheep and goat are closely related mammalian groups; the presence of Cu in goat LP and sLP may be a distinctive character of LP in this group, though their binding sites may be different. However, further studies are necessary to determine the exact binding site for copper and its possible structural role in the enzyme.

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