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Electrospray mass spectrometric study of haem changes during peroxidase denaturation

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Electrospray mass spectrometry (ESMS) was used to investigate the denaturation of horseradish peroxidase (HRP) on heating the enzyme under neutral conditions. The rate of loss of the haem signal followed closely the rate of peroxidase inactivation, strongly suggesting that the inactivation of the enzyme was due to haem changes. The results from previous work have indicated that the haem remained protein bound. It was therefore surmised that the haem had formed stronger, perhaps covalent, bonds with the protein during heating. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

ESMS is a soft ionisation technique which allows accurate and sensitive analysis of a wide range of analytes from low molecular weight compounds [less than 200 Daltons (Da)] to high mass biopolymers, for example proteins larger than 100 kDa (Niessen & van der Greef, 1992). In ESMS the sample in solution emerges, at atmospheric pressure, from a high voltage capillary into a strong electrostatic field producing an aerosol of highly charged particles. Evaporation of the solvent from these droplets results in sample ions. Proteins produce a series of multiply charged sample ions whose m/z ratios can be transformed to give a molecular weight profile.

During heat denaturation of peroxidase, alterations in the haem prosthetic group are considered to have an effect on the lipid oxidation activity of the denatured enzyme (Adams *et al.*, 1996). ESMS has a major advantage over ultra-violet/visible spectrometry methods for determining haem in peroxidase, in that it specifically identifies and measures the amount of haem without interference from denatured haemoprotein. It was therefore our objective to use ESMS to study the effect of heating on the haem changes in peroxidase.

MATERIALS AND METHODS

Type VI HRP was purchased from Sigma Chemical Company and used without further purification.

Heat treatment of peroxidase

Solutions of HRP were made up at 0.025 mM peroxidase in 0.2 mM sodium phosphate buffer (pH 7.0). This stock solution was separated into two sample batches. Each batch was heated separately on different days. After heating for up to 5 h in screw-cap borosilicate tubes in a thermostatic bath at 70°C, the solutions were rapidly cooled and assayed for peroxidase activity.

Peroxidase assay

Peroxidase assays were performed using hydrogen peroxide and guaiacol as described by Adams *et al.* (1996).

Electrospray mass spectrometry

All electrospray mass spectra were acquired using a Fisons Instruments VG Quattro (Type I) Triple Quadrupole Mass Spectrometer. The conventional electrospray source used a capillary voltage of 3.11 kV, a HV Lens voltage of 0.50 kV and a cone voltage of 50V. Nitrogen drying gas (oxygen free, British Oxygen Company) was introduced at the interface at a flow rate of 250 litres per hour and was also used as the nebulizer gas at a flow rate of 20 litres per hour through the electrospray probe.

Samples were prepared for ESMS by diluting the peroxidase solutions (50 μl) with 0–20 per cent aqueous formic acid (50 μl), distilled de-ionised water (400 μl) and acetonitrile (500 μl, HPLC grade, Rathburn). 10 μl of each sample was introduced into the electrospray source by loop injection (Rheodyne 7125 sample injector), into a steady pulseless flow (12.5 μl per minute) of 50 per cent acetonitrile (HPLC grade, Rathburn) in water (distilled, de-ionised).

Electrospray data was acquired using the Multi Channel Analysis (MCA scanning mode) from m/z 500

to 700 Da (the haem region) or 1700 to 2200 Da (the protein region), at a scanning rate of 2 or 4 seconds per scan over a seven minute period post injection. To enhance the signal-to-noise ratio, the averaged spectra produced were smoothed (Savitsky Golay method, with a smooth number of 2 and a peak width of 2.75 Da) and background subtracted (polynomial order of 2, tolerance 0.010) using VG (Fisons) Mass Lynx software. To obtain molecular weight information for the protein, the enhanced spectra were centred (Centroid mode at 80 per cent, minimum peak width at half height of 5 channels) and transformed (resolution 0.1250, range 40000 to 50000 Da) using VG (Fisons) Mass Lynx software. The mass spectrometer was calibrated using a solution of horse heart myoglobin (Sigma, 0.005 mM containing 0.2 per cent formic acid, using 50 per cent acetonitrile in distilled de-ionised water as the solvent).

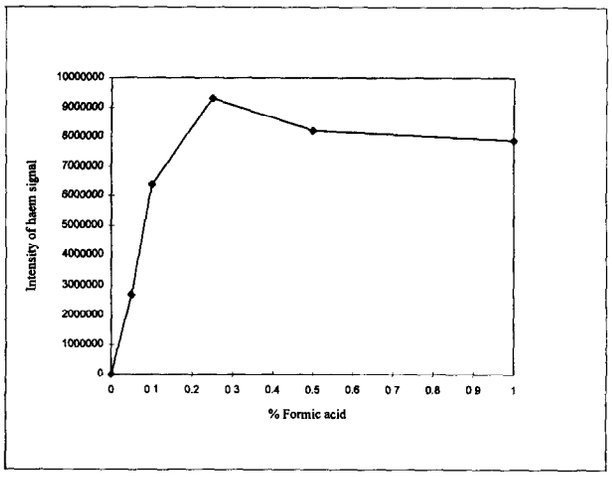

Fig. 2. The effect of formic acid concentration on the haem peak intensity in the ESMS spectra of native HRP at 50 V cone voltage.

RESULTS AND DISCUSSION

The electrospray mass spectrum of native HRP, showing both haem and protein regions is presented in Fig. 1. Transformation of the protein region showed the presence of five isoforms with similar molecular weights to those reported by Green & Oliver (1991).

Whilst ESMS cannot be used to measure the re-arrangement of the protein secondary structure during denaturation, changes in the amount of haem present can be readily studied. Formic acid is normally added to samples, for positive ion ESMS, to aid ionisation by protonating amino acid residues. In the case of haemoproteins, however, the acid also cleaves the haem from the protein (Ponce *et al.*, 1994). The amount of haem detected in native peroxidase was observed to remain roughly constant between 0.25 and 1 per cent formic acid (Fig. 2). Therefore 0.5 per cent formic acid was used in the samples containing heat denatured enzyme.

Peroxidase activities and haem intensities showed less than 5 per cent variation between the replicate heating experiments. The first order rate constant for the loss of the haem signal (0.19h $^{-1}$) was similar to that obtained for activity loss (0.20h $^{-1}$) (Fig. 3). This strongly suggests that the inactivation of peroxidase was due to haem changes.

Three types of change are feasible. One type involves the removal of iron from the protein-bound haem.

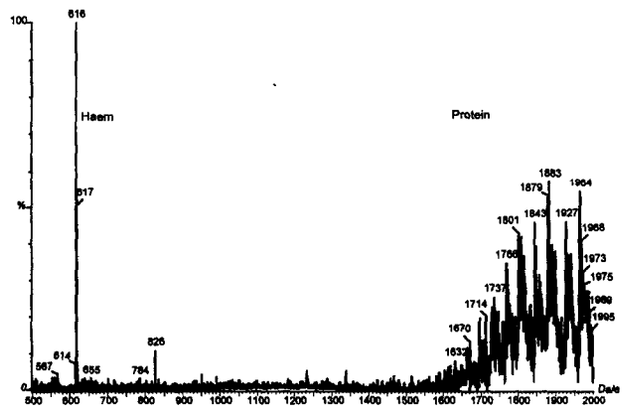

Fig. 1. Positive ion mode ESMS spectra of native HRP (Percentage base peak intensity versus mass/charge ratio)

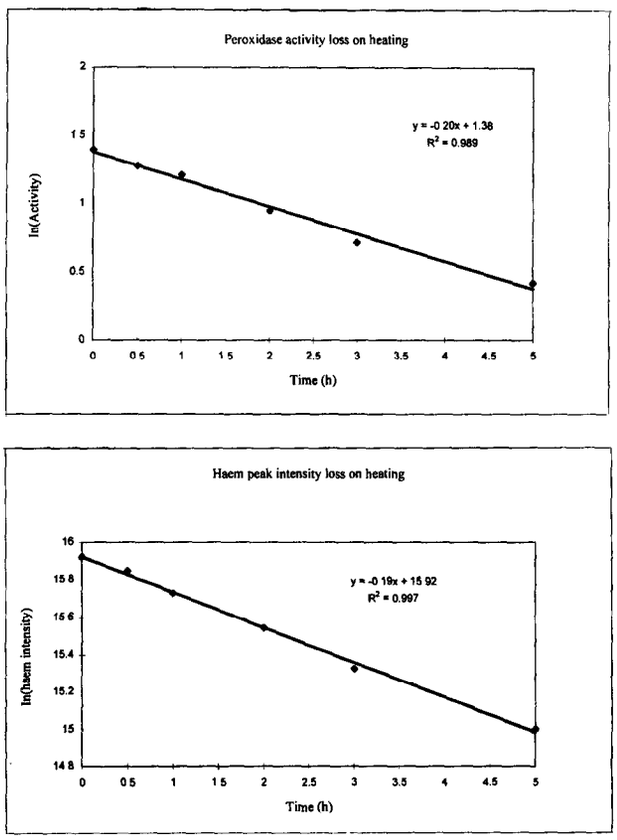

Fig. 3. Comparison of haem intensity and peroxidase activity losses on heating HRP at 70°C.

However, the broad spectrum of the heat denatured HRP found by Adams *et al.* (1996) was unlike the sharp, intense spectrum of the iron-free protoporphyrin-IX-apo-HRP complex described by Jullian *et al.* (1989). This suggests that the iron has not been released from the haem.

The second type of haem change involves the loss of haem from the protein and its subsequent degradation to compounds that are invisible to ESMS. Previous studies have demonstrated that the haem bonds to the protein are stable under neutral conditions. Using UV/VIS spectroscopy, Eriksson *et al.* (1971) found that heating HRP at neutral pH did not lead to a hypsochromic shift of the Soret absorption at 403 nm, which suggested that the haem was not cleaved from the enzyme. This was confirmed by Adams *et al.* (1996) who showed by the lack of haem dimer bonds in the circular dichroism spectrum on heating in phosphate at pH 7.0, that the haem remained attached to the protein.

The third type of haem change involves the chemical modification of the haem during heating and the formation of much stronger bonds with the protein that resist the acid-induced cleavage in the mass spectrometer. This type of haem change appears to be the most likely of the three types considered. New covalent bonds may have formed between the haem and the denatured protein though the nature of these remains open for further study.

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