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Renaturation of metmyoglobin subjected to high isostatic pressure

A. B. Defaye, D. A. Ledward, D. B. MacDougall & R. F. Tester

Department of Food Science and Technology, University of Reading, Whiteknights, PO Box 226, Reading RG6 2AP, UK

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Metmyoglobin solutions (0·2%) when subjected to pressures of 7·5 to 8·0 kbars at neutral pH were denatured and on standing only partially reformed the native state. The degree and rate of renaturation, as measured by resolubilisation, was very dependent on pH and ionic strength, suggesting that electrostatic forces dominate the protein–protein attractive forces in the aggregate. However, a marked temperature dependence indicated that hydrogen bond stabilisation of the aggregate could also be significant.

Spectral analysis of the solutions and precipitates suggested that the haem environment in the pressure-denatured state was typical of the ferric pigment of heat-denatured myoglobin. However, the initial colour of suspensions following pressure denaturation changed rapidly on standing and may indicate that the initial product of pressure treatment is a very unstable complex that rapidly converts to the denatured ferric pigment.

INTRODUCTION

It is almost a century since the first studies on the effect of high pressures on foodstuffs were described (Hite, 1899). Initial work on food was primarily of academic interest since commercial exploitation within the food industry was not then considered economically feasible. However, commercial vessels of several litres capacity and capable of withstanding pressures of several kbars are now available and this has stimulated research in the food area since the late 1980s (Farr, 1990; Johnston, 1992; Hoover, 1993; Knorr, 1993; Lechowich, 1993). Although most studies have looked at the effect of pressure on microbiological and enzymic stability there is increasing interest in the use of high pressures to denature and gel proteins or proteinaceous foods (Farr, 1990; Johnston, 1992). Denaturation by pressure will not necessarily yield gels similar to those formed by thermal treatment since hydrogen bonds and hydrophobic interactions behave differently when subjected to pressure than they do when heated (Morild, 1981). In addition, although electrostatic forces are not normally considered to be of major significance in aggregating denatured proteins in solution following thermal denaturation, they may be important in pressure treatment since pressure causes groups to ionise leading to a decrease in volume (Morild, 1981).

Several authors found that various proteins, under a range of conditions, denature irreversibly when subjected to pressure, forming gels or precipitates (Johnston, 1992; Knorr, 1993). However, Zipp and Kauzmann (1973) reported that the globular haem protein metmyoglobin under most circumstances is 100% reversibly denatured by pressure in dilute (~0·2%) solution. However, at around neutral pH, i.e. the isoelectric point of metmyoglobin (pK 6·9), some of the protein was precipitated following pressurisation. The nature of the precipitate was not studied. Since reactions leading to protein aggregation are important in many food systems the reversibility of the system and the nature of the precipitate were investigated.

MATERIALS AND METHODS

Materials

Metmyoglobin from horse heart (Lo + 61H7106) (Sigma Chemical Co.) was used without further purification.

The protein was dissolved in deionised water and pH was adjusted, if necessary, with 0·1M HCl or NaOH. Sodium chloride used was AnalaR Grade.

Pressurisation

Solutions were sealed in polyethylene sachets and subjected to pressures of 7·5 to 8·0 kbars for 20 min at

ambient temperature (Stansted Fluid Power Ltd, Stansted, UK). This pressure is in excess of that required to denature metmyoglobin under these conditions (Zipp & Kauzman, 1973).

Following pressurisation, samples were cooled from 25°–27°C and held at selected temperatures (± 1°C) prior to analysis. Most storage was at 4°C.

Analytical procedures

Extent of renaturation

Pressurised samples were gently shaken and filtered (0·45μ millipore) and the spectra of the filtrate recorded from 400 to 700 nm (Perkin Elmer Lambda 5 spectrophotometer). The spectrum of the original metmyoglobin solution was also recorded and the difference in absorbance at 505 nm used to assess the degree of renaturation.

Colour and reflectance spectra

Colour was measured on a Hunter Colorquest reflectance spectrophotometer. Spectra and CILAB L*, a*, b*, C* and h* colour values of the solutions and suspensions in the packages were obtained with a white tile behind the package 8 mm from the 19-mm diameter aperture of the integrating sphere. Although the effective path length of the transparent solutions was 16 mm it was considerably less for the suspensions because of reduction in transmission by light scattered by the denatured protein.

RESULTS AND DISCUSSION

Reversibility

Following pressurisation at around neutral pH, the metmyoglobin solution yielded a coloured solution and low-density precipitate which, following filtration, gave a solution spectrally identical to the original metmyoglobin and a reddish/brown precipitate. On storage at 4°C the precipitate in the solution dissolved slowly with time. The solution, however, retained its metmyoglobin characteristics indicating that the aggregated protein slowly renatured to metmyoglobin. Typical results for the rate of renaturation are shown in Fig. 1. Since precipitation occurred around the isoelectric point of the protein, not unexpectedly, dilute salt solutions (0·5 M) aided the renaturation (Fig. 1). Higher salt concentrations, though, led to stabilisation of the precipitate (salting out) (Fig. 2).

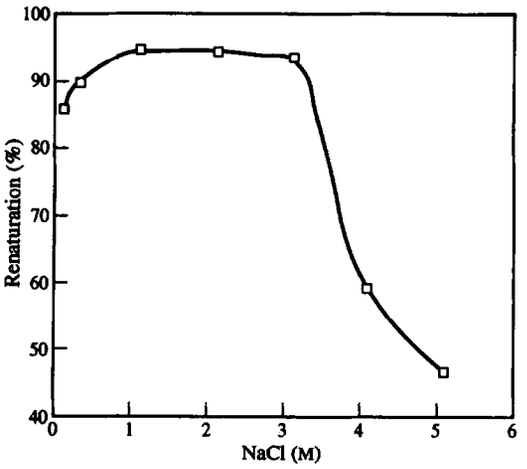

Fig. 2. Effect of salt concentration on the renaturation of 0·2% metmyoglobin at pH 6·9 pressurised to 7·5 kbar for 20 min and left to renature for 72 h at 4°C.

Adjustment of the pH (either before or after pressurisation) also affected the degree and rate of renaturation. For example, adjustment to 5·4 following pressurisation caused the precipitate to dissolve instantly to yield a solution of metmyoglobin. The effect of pre-pressurisation pH adjustment on the degree of renaturation after 72 h at 4°C is shown in Fig. 3. Pressure itself caused little change in the pH of any of the resultant solutions.

The rate of renaturation was very temperature-dependent, 50% renaturation occurring within 1 h at 30°C, compared to about 4 h at 10°C and 15 h at 4°C (Fig. 4).

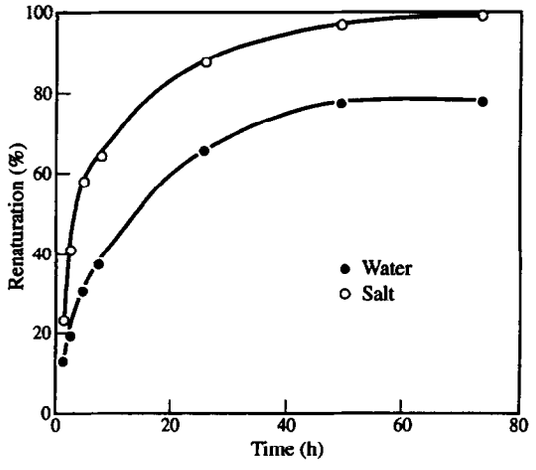

Fig. 1. Rate of renaturation of 0·2% metmyoglobin (●) at pH 6·9 in water and (○) in 0·5 M NaCl pressurised to 7·5 kbar for 20 min and left to renature at 4°C.

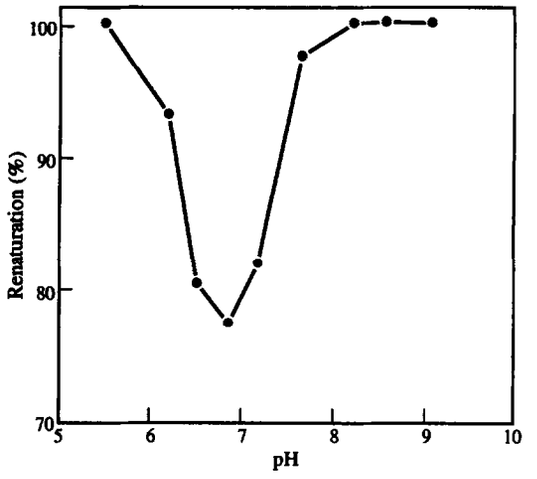

Fig. 3. Effect of pH on the rate of renaturation of 0·2% metmyoglobin pressurized to 7·5 kbar for 20 min and left to renature for 72 h at 4°C.

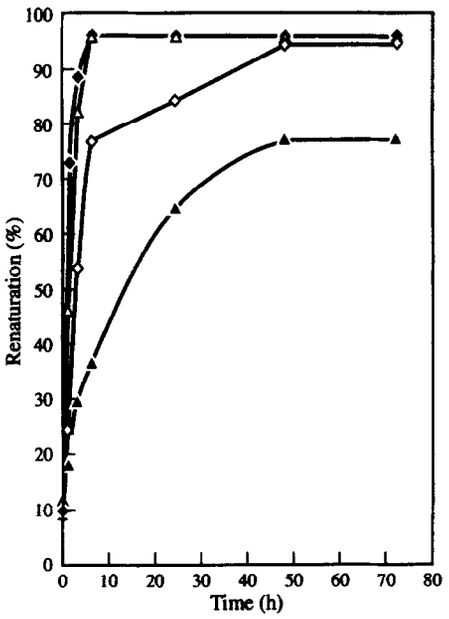

Fig. 4. Effect of temperature on the rate of renaturation of 0·2% metmyoglobin at pH 6·7 after pressurisation to 7·5 kbar for 20 min and then left to renature at (▲) 4°C, (◇) 10°C, (△) 20°C, (◆) 30°C.

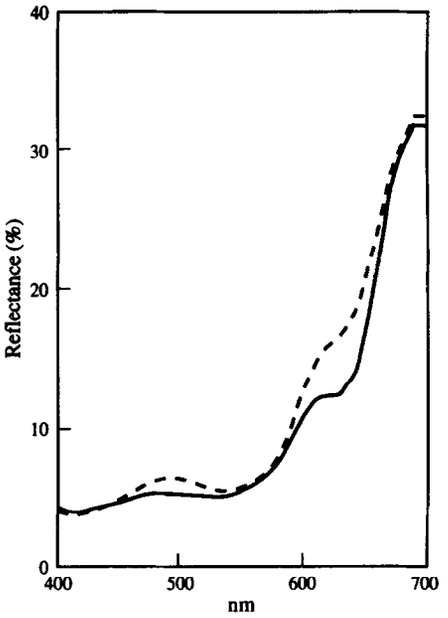

Fig. 5. Reflectance spectra of the turbid suspensions of 0·2% metmyoglobin recorded (– – –) 5 + (——) 20 min after pressurisation at 7·5 kbar for 20 min. The pH of the solutions was 6·8.

Nature of the precipitate

There is no evidence to suggest that, at pressures below those necessary to bring about unfolding of the polypeptide chains, the ligands bound to the haem change (Morishima & Hara, 1983). Typical progress of the changes in spectra and colour followed the sequence shown in Fig. 5 and Table 1. The spectrum of pre-treated brown metmyoglobin exhibited typical absorption at approximately 505 and 630 nm. After pressure treatment, the solution developed a pink 'baked bean tomato sauce' appearance. It was no longer transparent but had become a fine suspension which, upon standing, aggregated as it reverted to the initial brown of metmyoglobin and the solution became clear. Table 1 shows that the hue angle, h*, decreased from brown to red on pressurisation and then increased from red to brown. (Low values of h* are more red and higher values indicate loss of redness, i.e. developing yellow-brown). The pink precipitate from a pink cloudy suspension obtained on a Buchner filter pad after 5 min, was reddish pink and quite dense. After three days and reversion to brown, the precipitate was sparse. The effect of this is shown in Fig. 6, where the much higher reflectance after three days is due to decrease in precipitate and increase in reflected light from the filter pad.

All reflectance spectra can be seen to have distinct reflectance minima (absorption maxima) at about 412 nm and 535 nm with less-pronounced minima at about 630 and 570 nm. These spectra are typical of heat-denatured myoglobin (Ledward, 1971) and are characteristic of low spin ferric haemoproteins (Smith & Williams, 1970). The 5th and 6th ligands could therefore be imidazole groups (Ledward, 1971), the imidazole group displacing the water molecule at the 6th position during denaturation. The reverse occurs on renaturation/resolubilisation.

Although the reflectance spectra of the 'freshly pressurised' and 'aged' packages, and to some extent the corresponding precipitates, are not identical (and the colours vary) the haem environment may be very similar, i.e. diimadazole complexes, since the absorption peaks are consistent. The variations in colour are possibly due to the relative concentrations of the denatured and native forms (suspensions) or the density of precipitate on the filter pads. However, the possibility of a different, very unstable complex forming initially (per-

Table 1. CIELAB colour of the packages of (0·2% metmyoglobin, pH 6·8) pre- and post-pressurisation and the precipitate obtained on filtration. Pressurisation was at 7·5 kbar for 20 min

Sample	Time at 4°C after pressurisation	L*	a*	b*	C*	h*	Subjective appearance
Package	Pre-treated	43·9	19·6	24·1	31·1	50·9	Transparent
Package	5 min	34·0	17·8	12·5	21·8	35·2	Cloudy
Package	20 min	32·0	14·8	10·6	18·2	35·7	Cloudy, bigger particles
Package	2 h	43·6	19·5	26·8	33·2	54·0	Transparent with reduced ppte
Precipitate on pad	10 min	36·6	20·4	15·5	25·6	37·1	Reddish (dense)
Precipitate on pad	3 days	67·2	8·7	20·5	22·2	67·1	Brown (sparse)

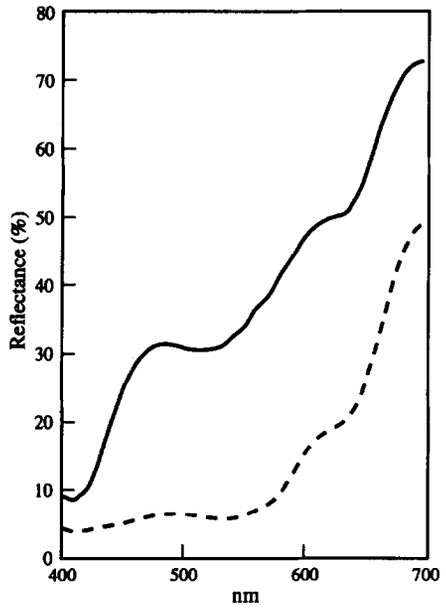

Fig. 6. Reflectance spectra of the precipitates obtained from 0·2% solutions of metmyoglobin at pH 6·7 following pressurisation at 7·5 kbar for 20 min and filtration after (– – –) 10 min and (——) 3 days at 4°C.

haps the reduced [ferrous] form of the denatured pigment) cannot be eliminated since the initial colour is different from that seen on storage and appears to change rapidly compared to the rate of resolubilisation (cf. Table 1 and Fig. 1).

Zipp and Kauzmann (1973) suggested that pressure denaturation of metmyoglobin is a two-stage process identical to that induced by heat, acid pH or urea. The present work would not refute this contention. However, the post-denaturational changes leading to aggregation at neutral pH must differ between the thermally and pressure-denatured states since the precipitates have different solubility properties. The heat-denatured form remains insoluble in water and dilute salt and can only be dissolved in such reagents as urea or sodium dodecylsulphate which disrupt hydrogen bonds and hydrophobic interactions or, at the extremes of pH (Ledward, 1971). The marked effect of pH (and to some extent dilute salt solutions) on the stability of the pressure-denatured aggregate suggests that electrostatic forces may be of paramount importance in dictating protein–protein aggregation under these circumstances. However, the marked temperature dependence suggests that hydrogen bonds, which break endothermically (electrostatic interactions have little temperature dependence and hydrophobic interactions over this temperature range would increase in strength) may also stabilise the aggregate.

As high pressure technology is increasingly being advocated as a method of food preservation/processing the interaction between pressure-denatured proteins and themselves, or other components in the food must be further pursued since the implications for such eating qualities as texture and juiciness may be significant. In addition the different modes of aggregation of biopolymers when subjected to high pressures may provide a valuable means of generating novel textured products.

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