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EFFECT OF HEATING TIME AT HIGH TEMPERATURE (145^{O}C) ON α_s-CASEIN and β-LACTOGLOBULIN POWDERS AT A RELATIVE HUMIDITY OF 95%

[35]

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ABSTRACT

Lowry method was applied to measure the solubility of α_s-casein and β-lactoglobulin protein in solvent system consisting of sodium dodecyl-sulphate (SDS) and β-mercaptoethanol (ME). The approach was applied to α_s-casein and β-lactoglobulin heated at temperature (145^{o}c) at 95% relative humidity.

The solubility of α_s-casein in water and the solvent mixture as a function of heating time in the RH 95% environment was high and increased with increasing heating time. In contrast β-lactoglobulin showed a zero solubility in water at all heating time and very low solubility in mixture of solvents. The differences between the two proteins are apparent even after short (20minutes) heating time. The color results of both proteins indicated that the darkening increased with increasing heating time. Heated α_s-casein had a wide molecular weight distribution range.

This was evident from both SDS-PAGE and the GPC/Malls –RI systems.

INTRODUCTION

Many food processes involve heat treatment of protein. Examples are pasteurization, heat sterilization, dehydration, extrusion and thermal gelation (**Mohammed, *et al* 2000**). Heat treatment can result in protein denaturation and subsequent association that causes changes in the functional properties of proteins (viscosity, gelation, solubility, emulsifying ability, foaming capacity, etc). Heat treatment generally causes a reduction in protein solubility. This is experimentally measurable property that can yield information about functional behavior as well as the physicochemical nature of the protein.

Under severe conditions, a reduction in solubility can be a consequence of the formation of covalent crosslink's. This crosslink formation is a function of a number of factors. These include, time and temperature of heating, protein structure type, moisture content, and the presence of other materials, e.g. reducing sugars. (**Mohammed 1995**) Little had been studied regarding changes to solid protein heated at high temperature.

Protein systems generally have a high solubility in solvent mixtures, such as β-Macaptoetheanol (ME) plus sodium dodecylsulphate (SDS) (**Hager, 1984**).

ME and SDS are generally considered to be capable of breaking respectively disulfide bonds and non-co-valent interaction. The objective of this work was to study the solubility behavior of α_s-casein and β-lactoglobulin in ME+ SDS when subjected to severe heating at low moisture contents and related this to its structural features.

In order to understand the solubility results of the heated and solubilized α_s-casein, an attempt was made to characterize the molecular size using sodium dodecyl-sulphate polyacrylamide gel electrophoresis(SDS-PAGE) and a gel permeation chroma-tography-malty angle laser light scattering(GPC-MALLS-RI)system.

MATERIALS AND METHODS

Materials

α_s-casein (c-7891) and β-lactoglobulin (L.8005) were obtained from sigma chemical Co. limited (Poole, Dorset). Chemicals, folin Ciocalteanreagent and β-mercaptoethanol (ME) were purchased from the sigma chemicals Co. Ltd, (Poole, Dorset).

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All other chemicals were obtained from fisons PLC (Loughbrough, UK) and were of analytical grade.

Methods

Three replicates were used for each test for α_s-casein and β-lactoglobulin. Each powdered sample was placed in an aluminum dish and the moisture content was determined by drying for six hours in a vacuum oven at 70°c. Dried samples were then adjusted to different moisture contents by equilibrating of 1g of each sample above a saturated salt solution of potassium nitrate, at RH of 95%. No mold growth was observed on any of the samples. All samples were then heated at 145°c generally for sixteen hours, variation in the duration of heating are given in the results section.

Heating was carried out in a forced air drying oven (Gallankamp.Loughborough, UK), ambient air was used (20±2°c) samples (1g) were heated using 2 regimes:

Dry heating; the dried samples (1g) without moisture equilibration were placed in unsealed glass ampoules (3ml capacity).

Wet heating; The moisture equilibrated samples (1g dry weight) were sealed in a3- ml glass ampoules.

Solubility measurement; protein solubilities in water and mixed solvent consisting of 1% SDS plus 1% ME were determined as follows; For the protein/solvent mixture, good linearity was found for protein concentration versus absorbance (R_2>0.99). Values obtained had standard deviation of <4% for all calculated protein solubility values using three replicate samples. Extensive preliminary work was carried out to ensure that there was no significant interference with the protein assay from SDS and ME at the dilutions used. Using the dilutions established, A 0.1g as test samples was removed from each powder sample and was mixed with 10ml of the solvent. 0.4ml of each supernatant was diluted to 10ml with water and the protein solubility determined using Lowry method. The results were expressed as a percentage of the total protein present in the sample.

Color evaluation

The color of the heated protein powder samples was determined using the Hunter Lab. colorquest spectrocolorimeter SnC5330 (Virginia, USA). Few grams of the heated protein powder samples were wrapped in cling film, to give a flat surface. The wrapped sample was then carefully positioned and clamped in front of the light. The depth of powder was such that when the background was altered from white to black, no variation in the L* reading occurred. This was checked for each sample. Reading was taken with illumination D65, observer angle 2 and specular inclusion. Reflected light was measured and displayed according to the L*, a*, b* color scale.

Before treatment, all the samples had L* values excess of 85%, where 100% equals to “white and 0% equals black.

Molecular weight determinations were made on heated samples after solubilization in water and the mixed solvent (SDS+ME) was sodium dodecyl sulphate-polyacrylamide gel electrophosis (SDS-PAGE) under denaturing conditions. Also runs on the gel were standards. These molecular weight markers (**Sigma, 1993**) were myosin (Rabbit muscle, 205KDa), β-galatosidase (E.coli, 116KD), phosphorylase b (Rabbit muscle, 97KDa), BSA (66 KDa) egg albumin (major component ovalbumin, 45KDa) and carbonic anhydrase (Bovine Erythrocytes 29KDa). Gel permeation chromatography coupled with multi angle laser light scattering(GPC/MALLS-RI system **(Fig. 1)** was used to measure of the molecular weight distribution of pure protein after heating and solublization would provide useful information about the degree of covalent cross-linking occurring during the heating process.

The system consisted of an on-line degasser (Degasser, DG-1200, uniflow,HPLC technology, Macclesfield, UK); an HPLC pump (Model 590 programmable solvent delivery Module, Waters, Millipore, Watford, UK) to pump the eluent through the system at a flow rate of 0.8 ml/min for all samples: a rheodyne injector model 7125 (Rheodyne,Inc. Cotati, CA, USA) fitted with a 100µ1 injection loop, a multi- angle laser light photometer (Dawn F,Wyatt technology Inc, Santa Barbara, USA) with a He-Ne laser operating at 632nm consisting of 15 detectors ranging from 22°c. to 160°c. and a waters 410 differential refractometer (Waters Associates Instruments Ltd., Northwich, Cheshire, UK).

This column system consisted of one PSS hema Bio Linear and one PSS Hema bio 40 column (both columns were obtained from polymer standard Service GmbH, Mainz, Germany).

Figure 1. Gel permeation chromatography coupled with multi angle laser light scattering GPC/MALLS-RI system

Column dimensions were 300 X 7.5mm, with the first column BSS Hema Bio Linear) having an exclusion limit for dextrans of 7 x10^6 Da and the second column (BSS Hema Bio 40) an exclusion limit for dextrans of 40 000 Da. The macroporous gel matrix of both columns consisted of acopolymer of hydroxyethymethacrylate across linked with ethyleneglycoldimethacrylate and modified with polar groups to improve the resolution of hydrophilic bio-polymers. The analytical columns were connected in the following order; PSS Hema Linear->PSS >PSS Hema Bio 40 and protected by a guard column (Ultrahydrolgel Guard Column). Void volume and total permeation volume of the column system were found to be 9.2 ml and 21.4ml respectively.

The apparatus settings were as follows

Protein standard and α_s-casein	2mg/ml
Flow rate	0.8ml/min
Temperature	Ambient
Injection volume	100µl
Eluent	phosphate/chloride buffer (pH6.8, 1=0.1) and 0.02% sodium azide

The complete system with all the required columns was equilibrated over night with the column eluent at ambient temperature before use. Phosphate /chloride buffer at an ionic strength=0.1 and pH 6.8(Green, 1933) was used as the column eluent. Sodium azide (0.02%) was also added to the solution.

10 mg were dissolved in 5ml of phosphate /chloride buffer immediately prior to experimental measurements. The final concentration of the samples was 2mg/ml. The samples were filtered through 0.45 µm membrane filters (Millex-HV, Millipore, and Watford, UK) to remove any particulate matter.

RESULTS

A- Solubility

Figure (2) shows the solubility of α_s-casein and β-lactoglobulin in water and the solvent mixture as a function of heating time in the RH 95% environment.

The α_s-casein is almost completely soluble in the solvent mixture after heating at 145^{o}c. Interestingly the solubilities in water was high and increased with increasing heating time. B lactoglobulin showed a zero solubility in water at all heating time and very low solubility in the mixture solvent.

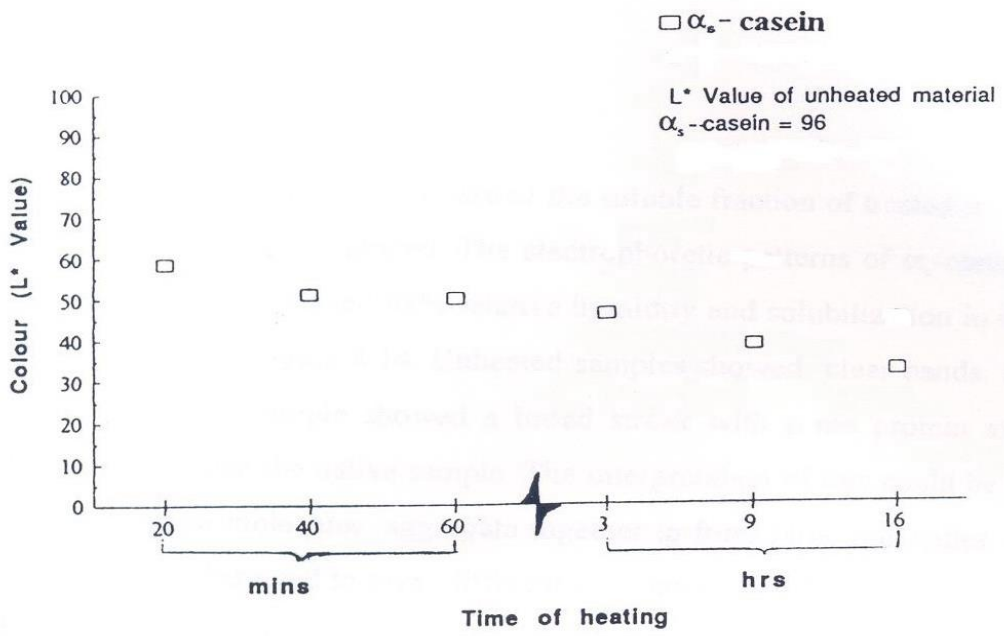

Figure 2. Solubility of α_s-casein after being heated at 145°c (RH=95%) for different times. Protein solubility was measured by the modified Lowry method in mixed solvent (1%SDS+1%ME) and in water.

B- Colour measurement

To obtain information about the changes that could be occurring during heating, the colour of α_s-casein showed were lower* values which means that the browning was greater with the prolongation of heating time to sixteen hours in **Figure (3)**. Browning under these conditions was greater for α_s-Casein than β-lactoglbulin.

C- Estimation of Molecular Weights

The variation in the solubility of the samples after extensive heat treatment could reflect the differences in the protein structures. For examples α_s-casein has very few bisulphate bridges. It is possible that the more open structure of the casein allows the prolonged heat treatment at elevated temperatures to break the backbone of the coiled protein. If this is the case changes in the molecular weight should occur. It could be expected that initially the apparent molecular weight of the whole sample should increase as more linkages occur and insolublization takes place. Breakage of the backbone allows the small pieces to become soluble; therefore this resolublized fraction should show a different molecular weight distribution to that of the native protein. To test whether this occurred it was decided to characterize the soluble, therefore this resolublized fraction should show a different molecular weight distribution to that of the native protein. To test whether this occurred, it was decided to characterize the soluble portion of α_s-casein after heat treatment. Two methods of characterization were used. (electrophores PAGE-MALLS-RI).

D- SDS-PAGE

The electrophoretic pattern of α_s-casein before and after heating at 145°C and 95% relative humidity and solublization in the mixed solvent are shown in **Figure (4)**. Unheated samples showed clear bonds. However, the heated α_s-casein samples showed a broad streak with some protein at a lower molecular weight than the native sample. The interpretation of this could be that after heating, the protein molecules aggregate together to form large molecules and then the molecules are disrupted to give different size ranges. The large molecules are not soluble, or if soluble they may be so large that they remain at the origin. The lower molecular weight fractions only were observed and this indicated that there was a decrease in molecular weight compared to the native protein. It was however, difficult to get quantitative

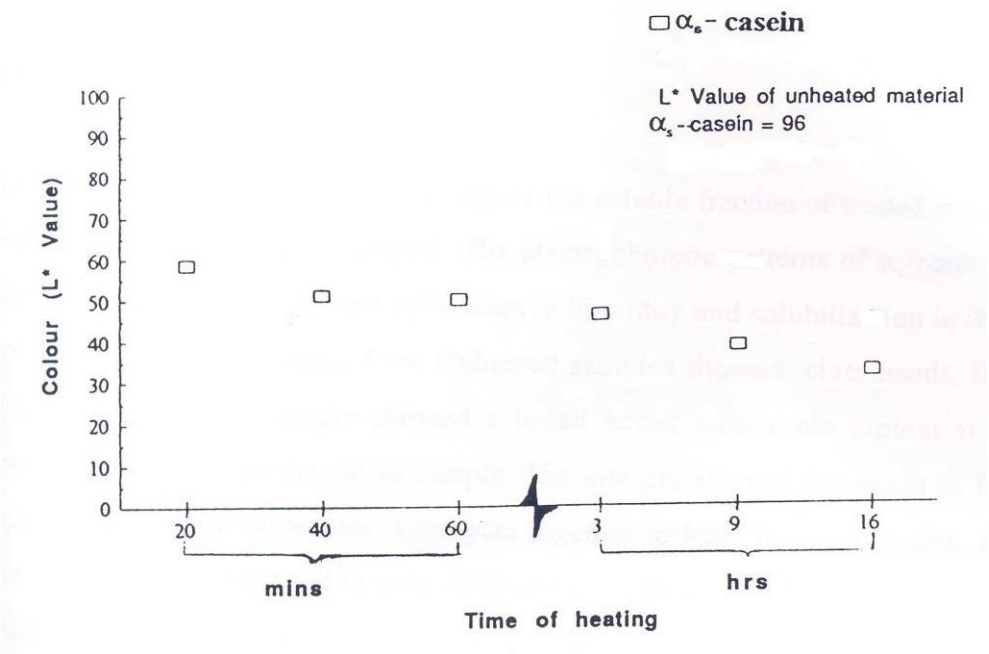

Figure 3. Effect of heating time on color of α_s-casein (RH95%) heated at 145°c as measured by a Hunter Lab Quest sphere spectrocolorimeter Sn C5330.

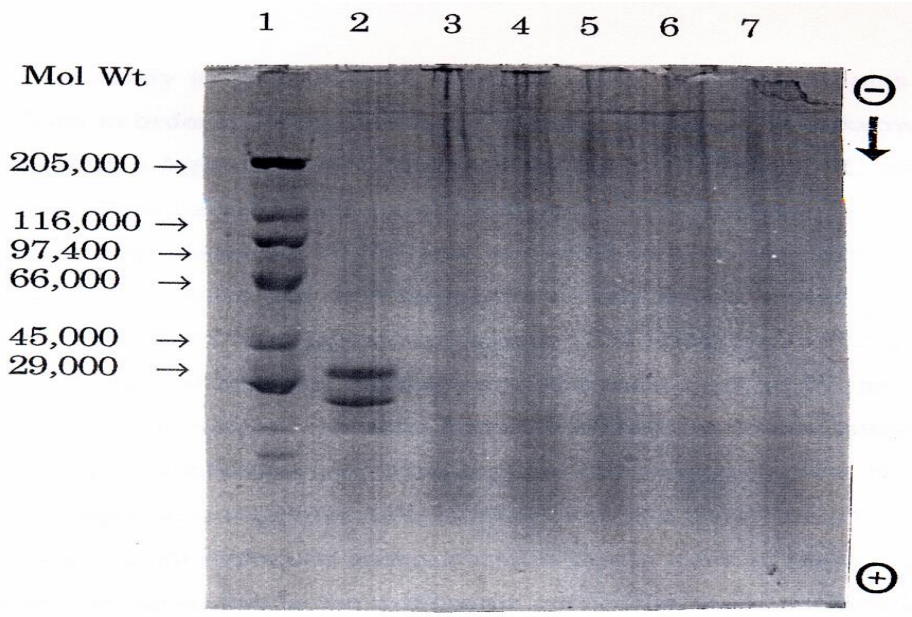

Figure 4. Polyacrylamide gel electrophoresis of the soluble portion of α_s-casein. Samples at 95% RH were heated at 145°c for various times and then solubilized in mixed solvent (SDS+ME). The lanes were(1) molecular weight markers, (2) unheated samples, samples heated for (3) 20min, (4)40min, (5)60min, (6) 9hours, (7) 16 hours.

information on the molecular weight distribution from patterns of this type and therefore an attempt was made to characterize the heated α_s-casein using gel permeation chromatography combined with multi-angle light scattering (GPC/MALLS).

C-GPC-MALLS-RI system HPSEC Method

The basic principle behind GPC-MALLS-RI System is the separation of molecules according to size. The sample components migrate through the column at different velocities and elute separately from the column at different times. Molecules are separated within a particular size range between the void volume and the total permeation volume. Large molecules elute first, small molecules elute later.

It is normally necessary to calibrate SEC columns using standard samples of known molecular weight in order to determine the molecular weight of an unknown sample.

If however, there is a light scattering detector on the end of the GPC column, it is possible to obtain absolute molecular weight and avoid the need for calibration. In this case however the light scattering signal was too small to give any information. This reflects the low concentration and lower molecular weight of the eluting proteins. The results **(Figures 5 and 6)** shows that heating α_s-casein (RH 95%) at 145^{o}C produces an increase in the amount of materials eluting to a main peak at 19ml.As heating time were increased, the amount of soluble materials eluting at lower volumes would indicate an increase in molecular size. This might be due to aggregation. However, the solubility of α_s-casein also increased with heating time and it might be possible that the observed increase of high molecular size material were due to more of the α_s-casein (23000M_w). This indicates that a mechanism other than size exclusion might be operating. The explanation for the 19ml peak found for α_s-casein (reported M_w23000) could be that the materials were binding to the column. This could be due to the hydrophobic properties of casein. Therefore although differences in elution pattern could be observed for the α_s-casein after heat treatment it is difficult to come to any firm conclusion as to the size of the soluble molecules.

DISCUSSION

From illustrative data **(Weisser, 1985)**, we would expect the water content to be around 0.4gH_2O dry solids for 95% RH. It had been reported that heating protein at low moisture content results in the generation of some additional water **(Oates *et al* 1987 and Yoong *et al* 1994)** this might have occurred to some extent in this work, but no measurement of this were made.

The major interest of this study is to clarity the effect of heating on protein powder. The obtained results could be summarized as follows:

(i) Heating resulted in a darkening of the powder as evidence by the decrease in the Hunter L* value. This darkening increased with increasing heating time.

(ii) The solubility behavior of the protein differed markedly.
In β-lactoglobulin there was a decrease in solubility with increasing heating time.
B-lactoglobulin had a very low solubility when heated for a long time at 145^{o}c.

(iii) The limited work on determining the molecular weight distribution of heated α_s-casein gave some what inclusive results. It was however clear that the heated protein had a wide molecular weight distribution range. This was evident from both

SDS-PAGE and the GPC/MALLS

What reactions are responsible for the insolublization, browning and subsequent resolublization? And how does the behaviour on heating relate to the composition and structure of the protein? It seems probable that two classes of reaction involved, both of which increase in rate with increasing heating. These classes are the reaction leading to insolubilization and reaction leading to solublization.

The formation of the additional cross links could be expected to render heated protein less soluble and as many of the proposed cross links utilized lysine, it could be expected that there might be a reduction in this amino acid. Loss of lysine and solubility, in a range of solvents, had often been used as monitors of changes in heated proteins, isopeptide bond formation between amino acids that had free amino groups. These include lysine, arginine, histdine, asparagines and glutamine. Of these lysine is likely to be the most important because of the greater availability of the amino group which is at the end of a carbon chain.

β-lactoglobulin, when isolate, BSA and egg albumin form thermal gels that do not melt on heating are the proteins that are the most difficult to resolubilize. It therefore seemed that the region of the proteins where they interacted to form heat

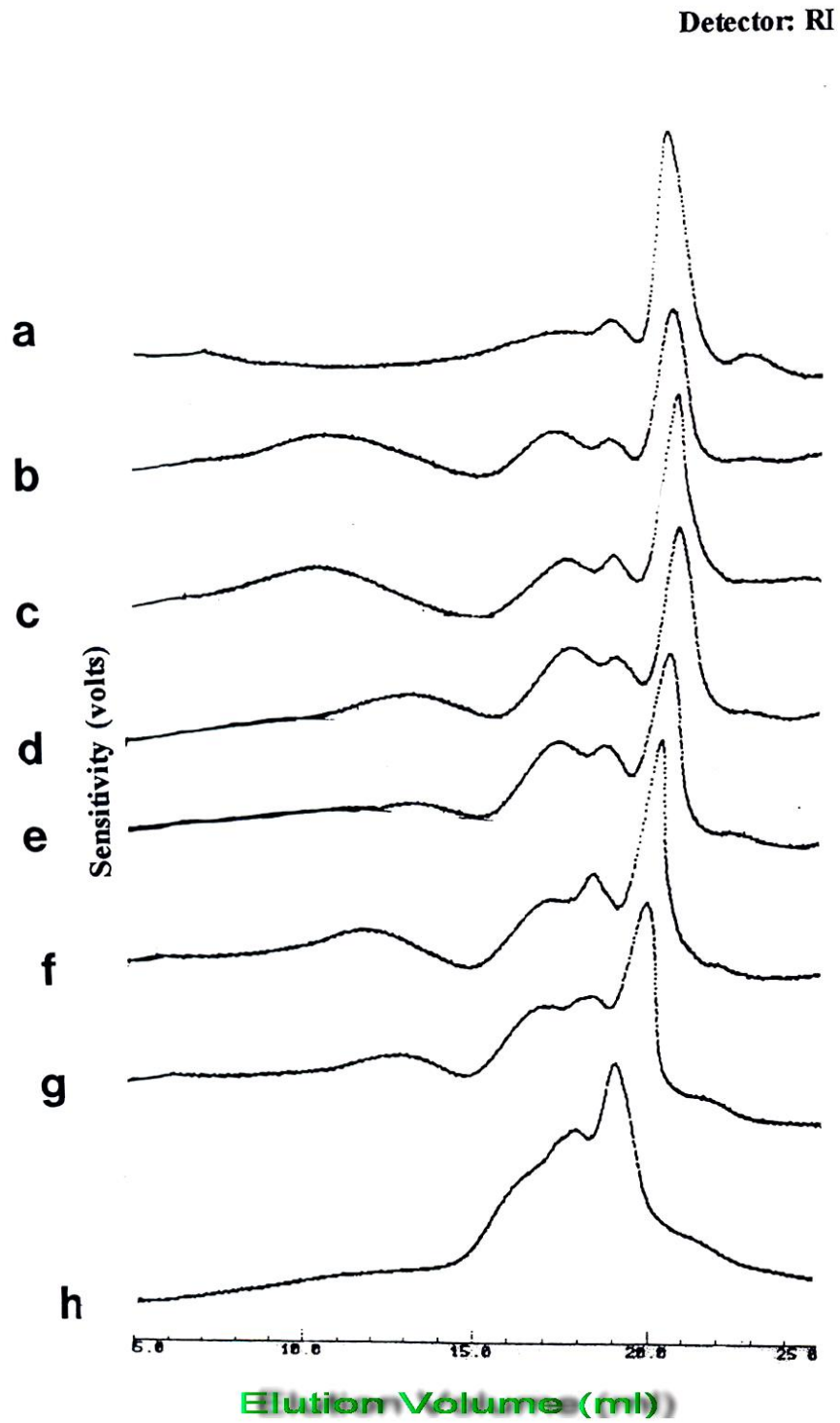

Figure 5. RI chromatograms showing the α_s-casein samples (RH95% at 145°c). At different time (a) native, (b) heated for 20min,(c)heated for 60min, (d) heated for 3hrs (e) heated for 9hrs (g)heated for 12hrs,(h)heated for 16hrs.

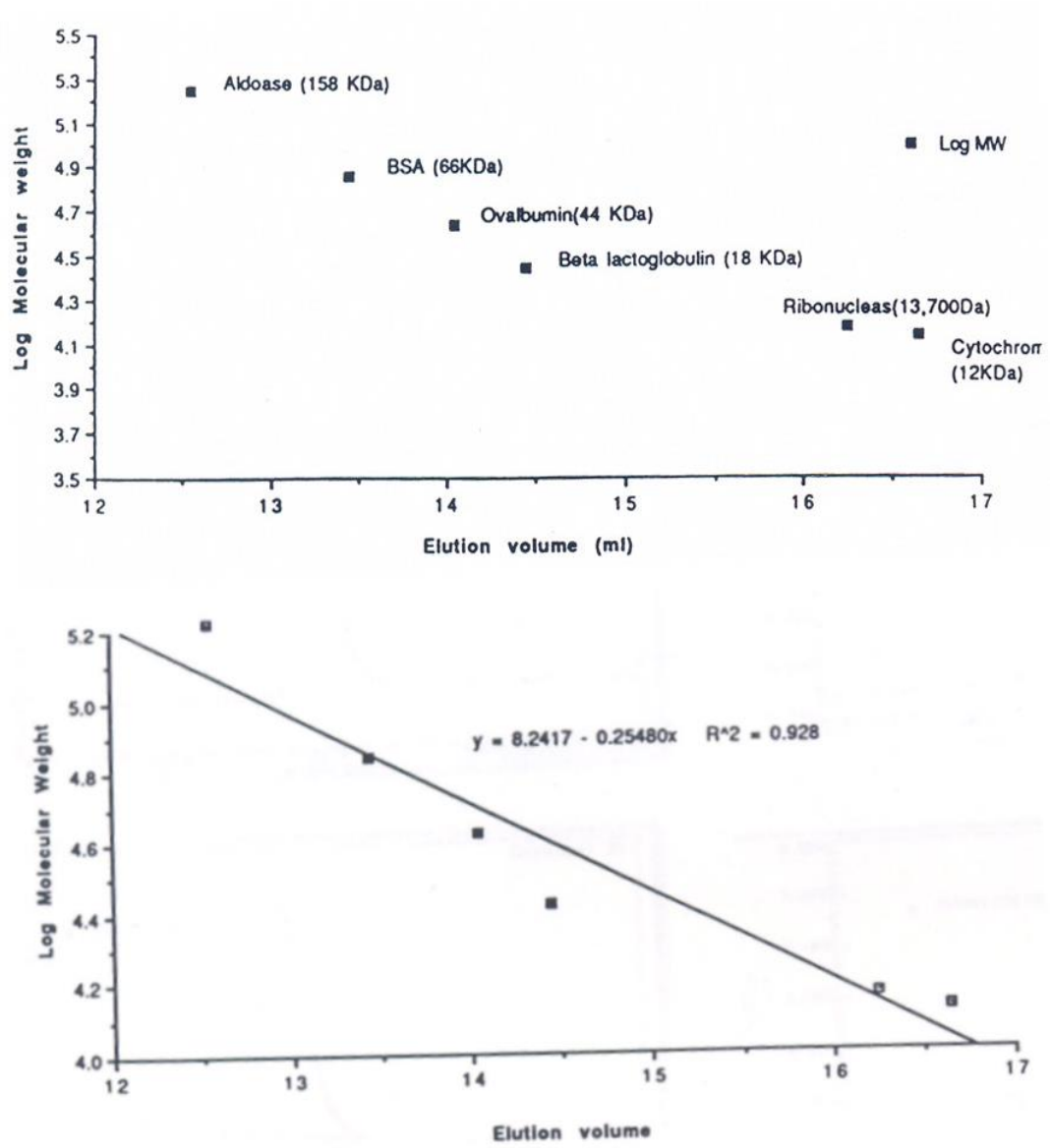

Figure 6. Relationship between the molecular weight and elution volume, showing the linear range in the analysis.

stable gels are the source of non- disulphide covalent cross links. These will form between the chain that are initially in close proximity as a result of the formation of conventional bonds (non-covalent and disulphide). The level of these will be less in the protein that does not gel on heating **(Cabodevila *et al* (1994)**.

It had been suggested that these covalent linkages could occur in regions that are linked by S-S bonds. In α_s-casein molecular weight of 23,000 Daltons), the proline+hydroxyproline is 8.39/16g nitrogen, while its cysteine content/16nitrogen is zero **(Morr and Ha, 1993)**. This is a measure of the possibilities for disulphide formation either by rearrangement of existing bonds or the formation of new ones. It could be seen that there is a rough correlation between the possibilities for disulphide bond formation as evidenced from the cysteine content and the temperature at which the protein starts to resolublize.

It is of great interest to see whether there is any other of the amino acid structure that relate to the solubility behavior.

Table 1. Displays the proline and hydroxyproline content along with the molecular weight of the protein of interest.

Table 1. Molecular weight, proline+hydroxyproline and cysteine plus ½cystine content of protein studied

Protein	Molecular weight(M_w), Daltons	Proline+hydroxyl proline 16 nitrogen	Cysteine plus½cystine 16 nitrogen
[1]α_s-lactalbuin	14,000	1.5	4.6
[1]β-lactoglobulim	18,300	5.1	3.4
[1]α_s-casein	23,000	8.3	0
[1]casein	19,000-24,000	10.6	0.34
[1]BSA	66,000	4.8	6.5
[2]Egg albumin	45,000	3.3	4.5
[3]Gelatin	10,000-200,000	26.8	0
[4]Gluten	40,000-10^5	11.8	2.8
[5]Soya isolate**	150,000 350,000	5.5	2.6

* The major groups are gliadin (average molecular weight: 40,000 Daltons) and glutenin (molecular weight from 10^5-10^7)

** The major protein are 7S (average molecular weight: 150, 00) and 11S with molecular weight approximately 350,000 Daltons

[1] Fox 1992, Morr and Ha, 1993

[2] Horns, 1980

[3]Maron, 1958, Ledward, 1989

[4] kinsella, 1982

[5] Hudson, 1982, Fox, 1992

It could be seen that three of the proteins that resolubilize easily (gelatin, gluten and caseinate) have high contents of these residues. Proline and hydroxyproline form unusual linkages within the polypeptide chain.

It is tempting to postulate that the linkages involving proline or hydroxyproline are the most heat labile. This would explain why gelatin cannot be used as a gelling agent in canned meat products subjected to high temperatures (120^{o}c) because of degradation.

The overall idea that emerges is that proteins which have an open random coil type structure which is partly a consequence of high levels of proline and hydroxyproline (residues that are incompatible with the α-helix) and a low level of disulphide bonds resolublize on heating. This is partly a consequence of the inability of proteins to form a high level of covalent non-disulphide links and also perhaps these are due to the liability of linkages involving proline or hydroxyproline. This is illustrated schematically in **Figures (7 a and b)**. Clearly, further work would be required to confirm this overall picture. Degradation might be the cause of the extensive browning found in α_s-casein heated at 145^{o}C. at 95% RH. Additional amino groups which could participate in browning would be created on degradation.

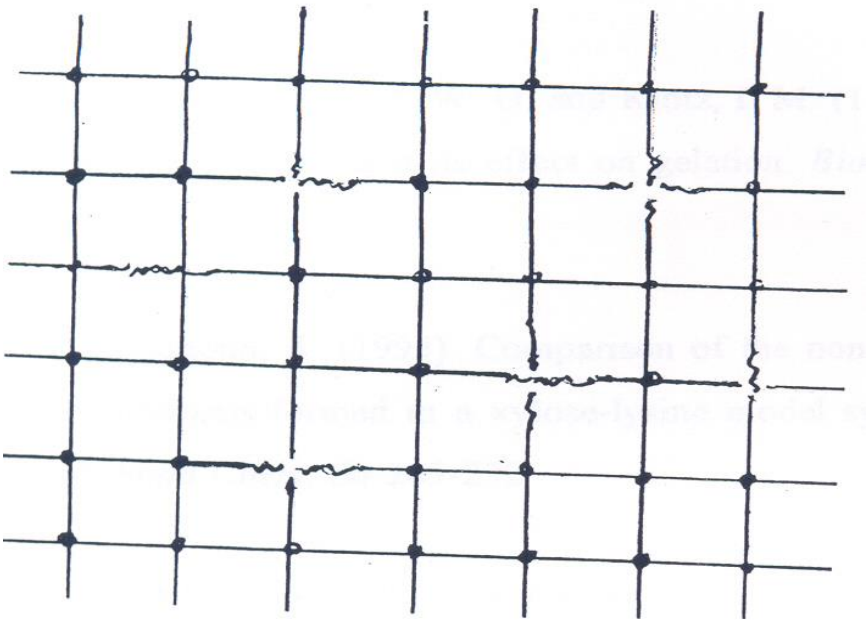

Figure 7a. High degree of non-disulphide covalent cross-linking initiating in the region of S-S linkages and a low degradation of proline and hydroxproline resulting in a low solubility.

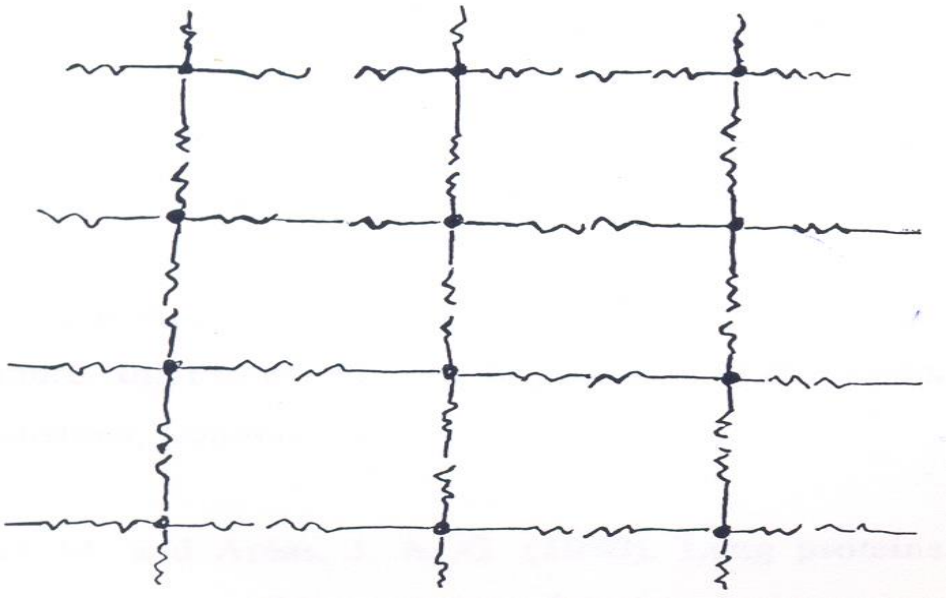

Figure 7b. Low degree of non-disulphide covalent cross-linking initiating in the region of S-S linkages and high degradation of proline and hydroxyproline resulting in a high solubility

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