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Application of the plastein reaction to mycoprotein: II. Plastein properties

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Abstract

Mycoprotein peptide hydrolysates have been used as starting materials for plastein synthesis. Compared to other proteins studied, mycoprotein hydrolysates were a relatively poor substrate for the plastein reaction and generally led only to thixotropic viscous solutions, rather than to gelled products, and only low yields of insoluble plastein material. Once formed, however, the insoluble fraction remained insoluble over the whole pH range of 2–11. In contrast to many other plasteins, the mycoprotein material was not solubilised by detergents such as sodium dodecyl sulphate although, like others, it was largely solubilised by 50% (v/v) organic acids or 1 M NaOH and partially solubilised by chaotropic agents such as 8 M urea, 6 M guanidinium chloride and 7 M potassium thiocyanate. A combination of 8 M urea and 50% (w/v) citric acid completely solubilised the plastein to a clear solution. Gel filtration failed to reveal any change in peptide molecular weight distribution on plastein formation while ion-exchange chromotography showed some quantitative differences but these were difficult to interpret as most of the material did not adhere to the anion-exchange column under the conditions used. Amino acid analysis revealed a marked preferential incorporation of hydrophobic peptides into the plastein fraction. Differential scanning calorimetry results showed only broad peaks which suggested heterogeneous reaction mixtures and products with no well-defined structural elements. These results are entirely consistent with plastein formation proceeding via a purely physical aggregation pathway.

Keywords: Quorn; Mycoprotein; Plastein; Reaction mechanism

1. Introduction

The plastein reaction has a long, but in many ways, unsatisfactory history in that the reaction appears to have potentially useful practical consequences but to date, these have been largely unrealised. For example, it has been suggested as a procedure both for removing the bitterness of protein hydrolysates and for generating desirable flavours (Fujimaki, Arai & Yamashita, 1977; Fujimaki, Yamashita, Arai & Kato, 1970; Yamashita, Arai, Matsuyama, Kato & Fujimaki, 1970); it can enhance the nutritional value of proteins deficient in essential amino acids (e.g. methionine, cystine, lysine) or reduce the content of others (e.g. phenylalanine) for dietetic applications (Fujimaki et al., 1977; Yamashita, Arai, Imaizumi, Amano & Fujimaki, 1979; Yamashita, Arai & Fujimaki, 1976; Yamashita, Arai, Tsai & Fujimaki, 1971) and, by producing viscous solutions and gels, it can greatly modify the functional properties of proteins and protein foods or be used to generate proteinaceous surfactants (Andrews & Alichanidis, 1990; Edwards & Shipe, 1978; Fujimaki et al., 1977; Shimada, Yamamoto, Sase, Yamazaki, Watanabe & Arai, 1984; Sukan & Andrews, 1982a, b; Tsai, Yamashita, Arai & Fujimaki, 1972; von Hofsten & Lalasidis, 1976; Watanabe, Toyokawa, Shimada & Arai, 1981). All this can be achieved without adding any non-protein ingredients or undesirable additives.

Why then, has the plastein reaction failed to catch the attention or interest of food manufacturers and processors or dietitians? The answer must be economics and the fact that many of the desirable goals can be achieved by alternative routes. For example, careful choice of ingredients and appropriate conventional processing leads to acceptable diets for dietetic applications, while blending and processing, and/or the addition of thickening agents, can be used to manipulate viscosity. The addition

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of proteins such as gelatin or polysaccharides is a cheap and acceptable method for producing gelled products. In order to be able to compete with such existing approaches, the plastein reaction must have some advantages and must preferably be no more costly. Advantages in addition to those referred to above, are likely to include the facts that as an entirely enzyme-mediated process the plastein reaction can be represented as "natural" and, since it takes place at relatively low temperatures, energy costs should be low. Only food-grade enzymes are needed and no solvents other than water are required. Procedures to control the extent of reaction or stop it when it has gone far enough are not usually necessary and the reaction conditions themselves should be reproducible and require no post-reaction treatment. A disadvantage is that the plastein reaction is a multi-step reaction, generally involving a concentration stage and pH adjustment between steps, which can add unacceptably to the cost. To date it has been applied principally to food ingredients and relatively pure model proteins rather than to intact food materials. It is not yet possible to predict the outcome of the plastein reaction except in general terms and, in fact, literature reports imply a rather variable response, depending largely on the identity of the initial protein substrate and the enzymes used.

Many aspects of the plastein reaction could be improved if the reaction mechanism itself was fully understood, as this would be likely to lead to an improved ability to predict and control product qualities and might also lead to ways of simplifying the reaction procedure, with consequent gains in terms of efficiency and cost. Unfortunately, in spite of considerable study there is no consensus of opinion about a single reaction mechanism. It is increasingly apparent that there may be no single definitive mechanism but rather various mechanistic pathways, leading to generally similar reaction products and changes in protein functional behaviour, with particular pathways being favoured by particular substrate proteins or reaction conditions. Generally, the reaction consists of hydrolysing a substrate protein enzymically with proteinases to a mixture of peptides, followed by concentration to generally about 20–40% (w/w) and re-incubating, following addition of a further batch of enzyme, which produces some form of insoluble or high molecular weight material in what is sometimes referred to as the plastein condensation.

In this paper, we discuss aspects of the mechanism of the plastein reaction and also report on some of the physical and chemical properties of plastein products prepared as described in an accompanying paper (Williams, Brownsell & Andrews, 2001) from Quorn™, a commercially available mycoprotein. This extends our knowledge of the reaction into a new class of substrate food proteins.

2. Materials and methods

For plastein synthesis, in a typical experiment 8 g of lyophilised Quorn peptide mixture (Williams et al., 2000) was mixed with 12.0 ml H_2O and 4.0 ml of 0.5 M sodium acetate pH 5.0 buffer containing 20 mg/ml of pepsin (E.C. 3.4. 23.1; pepsin 1:60,000, Sigma Chemical Co. Ltd. Poole, UK) The mixture was incubated at 37°C for 42 h, cooled to room temperature and used within 4 h of preparation. For some experiments, portions of 1.0–1.5 ml were centrifuged for 10 min at 13 000 rpm in a microcentrifuge to yield fractions of the supernatant and of insoluble plastein material as a pellet. In larger scale preparations of plastein and/or supernatant, centrifugation at 3000 ***g*** for 30 min was used. In all cases, the plastein pellets were washed free of adhering supernatant by at least two cycles of resuspension in H_2O to a volume equal to that of the initial sample and recentrifugation.

Trinitrobenzene sulphonic acid (TNBS) was from Sigma Chemical Co Ltd., Poole, UK. For the measurement of amino groups, 10 μl portions of sample solution were mixed with 1.0 ml of 0.1 M sodium borate buffer pH 9.4, 1.0 ml of 10% (w/v) sodium dodecyl sulphate (SDS) and 1.0 ml of 0.1% (w/v) of TNBS in H_2O. The mixtures were incubated at 37°C for 60 min and 1.0 ml of 0.5 M HCl added, before reading optical densities at 410 nm.

Gel filtration chromatography was performed on a Superose 12 FPLC (fast protein liquid chromatography) column (Pharmacia Biotechnology Ltd. Milton Keynes, UK) attached to a Dionex high-performance liquid chromatography (HPLC) system consisting of a DX-500 pump and Model AD20 UV detector (Dionex Ltd., Camberley, UK). Column flow rate was 0.7 ml/min with 0.1 M sodium phosphate pH 7.0 buffer containing 0.25 M NaCl used for elution. Sample volumes of 50 μl were used throughout, with concentrations generally being approximately 1–2 mg/ml. Plastein pellet material was largely solubilised in the phosphate buffer by addition of excess solid urea (to approximately 10 M). All samples were pre-filtered through 0.22 μm filters before application to the column.

Ion-exchange chromatography was likewise performed on a Pharmacia FPLC column; in this case a Mono Q anion-exchange column, attached to the same Dionex apparatus. Sample volumes were again of 50 μl, but in this case, portions of peptide hydrolysate or of plastein supernatant were diluted in H_2O to approximately 10 mg/ml while plastein pellets were largely dissolved in H_2O by adding solid urea to saturation (10 M+). All samples were pre-filtered before application to the column and elution was then performed with a salt gradient in 0.01 M sodium phosphate buffer consisting of 0–3.0 ml with no NaCl then a gradient from 3.0–23.0 ml rising linearly from 0 to 0.5 M NaCl, followed by 3 ml of 0.5 M NaCl and finally re-equilibration with 3 ml of zero-salt buffer. A flow rate of 1.0 ml/min was used throughout.

For amino acid analysis, samples of hydrolysate peptide mixture and plastein pellet and supernatant material were made up at 1.0 mg/ml in 6 M HCl containing 0.1% phenol, heated in vacuo at 110°C for 24 h and the residues evaporated to dryness. They were then reconstituted in 5 ml of pH 2.2 lithium citrate buffer containing 100 μmole/ml of amino ethyl cysteine as an internal standard. Portions of 50 μl were then loaded onto a Beckman 6300 amino acid analyser (Beckman Instruments Ltd., High Wycombe, UK).

Differential scanning calorimetry (DSC) was performed with a Perkin-Elmer DSC6 thermal analysis instrument (Perkin-Elmer Ltd, Llantrisant, UK). Samples of 8–10 mg of freeze-dried pepsin hydrolysate of mycoprotein, or of plastein pellet material which had been dried to constant weight in a desiccator over P_2O_5, were weighed into sample pans which were then purged with N_2, sealed and placed in the DSC instrument. The heating programme was over the range 20–400°C, increasing linearly at a rate of 5°C/min.

3. Results and discussion

3.1. The stability of plastein towards pH

For these experiments, mycoprotein plastein was prepared as described in Section 2 and 1.0 g portions of the resulting plastein-containing suspension were mixed in pre-weighed Eppendorf tubes with 0.4 ml portions of phosphate buffers of various pH values. These were prepared by making up a 1 M solution of Na_2HPO_4, which gave a pH of about 9.4, and adjusting portions to pH 10 and pH 11 with 2 M NaOH and to more acidic values between pH 2.0 and 9.0 using conc. HCl. The buffered plastein solutions were allowed to stand at 20°C for 24 h and centrifuged in a microfuge for 10 min at 13 000 rpm. The supernatants were carefully decanted off and the pelleted plastein washed three times by suspension and recentrifugation in 1.0 ml H_2O. Finally the plastein pellets were dried to constant weight (several days) over P_2O_5 in a vacuum desiccator. Plotting pellet weight versus pH (Fig. 1) showed that once insoluble plastein material had been generated at pH 5.0, it was unaffected by exposure, even for 24 h, to aqueous buffers within the pH range 2–11. The slight increase in solubility with increasing pH to 9.0, and sharp increase above that seen previously with casein-derived plastein (Sukan & Andrews, 1982b), was not observed here with mycoprotein plastein; pellet weight remaining constant over the entire pH range studied.

3.2. Plastein solubility

Pepsin-catalysed mycoprotein plastein was prepared as described in Section 2 and 1.0 g portions centrifuged at 3000 ***g*** for 30 min. The supernatant liquid was decanted off and the pellets resuspended in the reagents shown in Table 1. After a few minutes of stirring and then allowing to settle, the proportion of plastein solubilised was assessed visually (Table 1). As can be seen, organic acids such as citric and lactic were most effective at solubilising the plastein material, with acetic and formic acids slightly less so. Chaotropic agents such as 6 M guanidine hydrochloride and 8 M urea were also quite effective, with 7 M ammonium thiocyanate having a lesser effect. Salts such as phosphate and NaCl (data not shown) had no apparent effect and neither did inorganic acids (HCl) or bases (NaOH and NH_4OH) or detergents (Table 1).

In repeat experiments, pre-weighed Eppendorf tubes were used as above for the pH stability experiments and, after collection of the plastein pellets and three washes in H_2O by suspension and centrifugation, the plastein pellets were resuspended in the potential solubilising agents, then re-centrifuged, the supernatants separated and the pellets washed and dried to constant

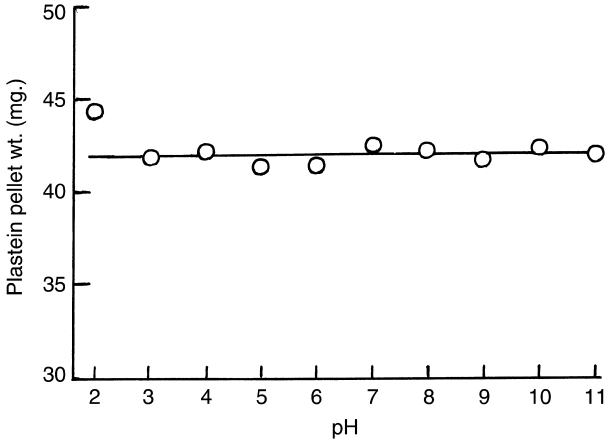

Fig. 1. The stability of mycoprotein plastein formed at pH 5.0 with a pepsin catalyst towards buffers of pH ranging from 2.0 to 11.0 (see text for details).

Table 1
Solubility of mycoprotein plastein in various reagents

Reagent	Qualitative evaluation
H_2O (Control)	No effect
6 M Guanidine hydrochloride	Solubilised
8 M Urea	Solubilised
7 M Ammonium thiocyanate	Partially solubilised
5% (w/v) Sodium dodecyl sulphate	Insoluble, precipitated
5% (v/v) Nonidet P4O	No effect
8 M NH_4OH	No effect
2 M NaOH	No effect
5 M HCl	No effect
50% (v/v) Acetic acid	Mostly solubilised
50% (w/v) Citric acid	Solubilised
50% (v/v) Lactic acid	Solubilised
50% (v/v) Formic acid	Partially solubilised

weight as above. The pellet weights are shown in Table 2. Portions of the supernatant fractions were assayed for peptide content using the TNBS procedure for measuring amino groups (Sukan & Andrews, 1982a) and, clearly, the more effective an agent was at solubilising the plastein, the lower would be the pellet weight and the higher the TNBS reading. Since plastein material is composed of many different peptides which would give different molar responses in the TNBS reaction, no attempt was made to calibrate the method. All that was required was relative readings to confirm the data given by the pellet weights, so the TNBS data is given only as comparative optical density readings in Table 2 and these were obtained by taking the same volumes (10 μl) of each supernatant for use in the assay. In general, it can be seen that high TNBS readings mirror low plastein pellet weights, but the relationship was not a precise one. Likewise, the data in Table 2 are generally consistent with the visual observations in Table 1. Clearly the organic acids at either 50% w/v, as in Table 1, or at 70% w/v, as in Table 2, were most effective at solubilising mycoprotein plastein, just as they are for casein-derived plastein (Sukan & Andrews, 1982b). However, Table 2 suggests that 1 M NaOH was at least as effective at solubilising mycoprotein plastein as the organic acids, as judged from pellet weights, and 2 M HCl nearly so. However, in neither case was this fully reflected by TNBS readings. Solubility in NaOH (but not HCl) was also a characteristic of casein plastein (Sukan & Andrews, 1982b). Rather less effective at solubilising mycoprotein plastein than organic acids were the chaotropic agents, which in this case paralleled the visual assessment, with guanidine hydrochloride being the most effective and potassium thiocyanate the least. Although the level used in the Table 2 data was very much lower at 0.3% than the 5.0% used with visual assessment, the anionic detergent, SDS appeared to have very little effect. None of these readings and comments, however, take account of other effects observed as a result of plastein treatment with these reagents. For example, NaOH addition led to a subtle change in the plastein mixture from being a rather viscous solution to a rather more gelatinous consistency. Conversely, both HCl and SDS led to a complete loss of any gel-like properties and a marked fall in viscosity, so that the resulting material behaved more like a conventional solution and rapidly sedimenting precipitate. Some of these physical changes may have masked the changes in solubility revealed by the data shown in Table 2 and may explain apparent discrepancies between Tables 1 and 2.

Table 2
Mycoprotein plastein solubility

Reagent	Pellet weight (% of control)	Trinitrobenzene sulphonic acid readings (absorbance at 410 nm)
H_2O (Control)	100	0.120
8 M Urea	86	0.656
6 M Guanidine hydrochloride	77	1.141
7 M Potassium thiocyanate	98	0.671
0.3% Sodium dodecyl sulphate	98	0.797
2 M NaCl	86	0.390
2 M HCl	52	0.660
1 M NaOH	42	1.372
70% Lactic acid	66	1.473
70% Formic acid	54	2.150
70% Acetic acid	53	2.311
70% Citric acid	44	2.447

It is worth noting, that under no conditions did the mycoprotein form plastein material with the thixotropic gel character observed with casein plastein formation but, rather, in all cases, led essentially to opaque viscous solutions which, while exhibiting thixotropy, never gave rise to a true gel state. It was also observed that while some of the above reagents largely solubilised the mycoprotein plastein, individually none of them completely solubilised it, as judged either visually or by pellet weights. In all cases, the best that could be achieved was opaque solutions that still threw down a precipitate on centrifugation. In a separate experiment, however, it was found that a combination of chaotropic agent, such as 8 M urea, with citric acid, completely solubilised the plastein to give a clear solution. Thus, as with casein plastein, the ability to solubilise the mycoprotein plastein under conditions that would not disrupt covalent bondings indicates a predominant role for physical processes such as hydrogen-bonding, electrostatic bonds and/or hydrophobic forces in the plastein synthesis mechanism.

3.3. Column chromatography

Column chromatography analysis was performed on both Superose 12 gel filtration and Mono Q anion-exchange columns, with monitoring at both 280 and 215 nm. This was done because monitoring at 280 nm is more specific for protein analysis, but relies on absorption by tyrosine or tryptophan residues which are both amino acids with relatively low abundance, so many peptides generated by the initial hydrolysis will contain neither of them, and hence have no significant absorption at 280 nm. Thus, the less specific wavelength of 215 nm was also used to give a more comprehensive picture. Samples of the initial mycoprotein hydrolysate, and of the centrifuge supernatant following plastein formation, and of the plastein pellet material largely solubilised by addition of urea to 8–9 M, were analysed in each case. Sample volumes applied to the columns throughout were constant (50 μl) but the sensitivity range of the HPLC detector was adjusted to give outputs of broadly comparable peak size. This facilitated a visual comparison, but rendered quantification difficult. However, since

individual peptides would have unknown and widely differing extinction coefficients and the peptide populations of the initial peptide mixture, plastein and supernatant fractions would be expected to differ, either qualitatively or quantitatively or both, it was considered that any attempts at quantification would be certain to be inaccurate. Thus the column analysis results were assessed only in quantitative terms, by comparing relative peak heights and positions, and by the absence or appearance of specific peaks between runs.

Fig. 2a shows the gel filtration results of the mycoprotein pepsin hydrolysate (peptide mixture) used as starting material for plastein synthesis and the subsequent supernatant and plastein pellet fractions following plastein formation with UV column monotoring at 215 nm, while Fig. 2b shows the same analysis with UV monitoring at 280 nm. The results at both wavelengths suggested that the largest material (peak A) and another peak (C) of relatively high molecular weight peptide(s) were relatively enriched in the plastein pellet fraction, whereas peak B (consisting of large peptide[s]) and the peaks D–H of smaller peptides remained essentially in the supernatant. There was no evidence for any substantial change in the size ranges of peaks (relative peak sizes) or for an increase in high molecular weight material as a result of plastein formation. The plastein pellet material was solubilised for this work by urea addition, which is not capable of disrupting covalent bonds, so the finding that the peak identities in the solubilised plastein were the same as in the starting peptide mixture and in the supernatant fraction underlines that the mechanism of plastein formation was principally a physical process, as found in earlier work with casein protein (Andrews & Alichanidis, 1990; Sukan & Andrews, 1982a, b). Interestingly, none of the peptide peaks seen at 215 nm were missing from the 280 nm profiles. This showed that, although all peaks are likely to be highly heterogeneous in terms of component peptides (given the complex nature of the mycoprotein starting material), they nevertheless all contained at least some peptide with a significant tyrosine and/or tryptophan content.

Fig. 3a, b shows the ion-exchange chromatography profiles with monitoring at 215 and 280 nm, respectively, using the Mono Q column of mycoprotein pepsin hydrolysate and then the pellet and supernatant fractions following plastein formation. As with the above gel filtration results, essentially all the peaks seen at 215 nm could also be identified in the 280 nm profiles, although in this case, there appeared to be some 280 nm peaks that were not observed at 215 nm. This anomaly was probably a consequence of the fact that by far the major proportion of mycoprotein peptide did not adhere to the Mono Q column under the reaction conditions used and passed directly through the column. Thus the peak profiles shown in Fig. 3 represent only a

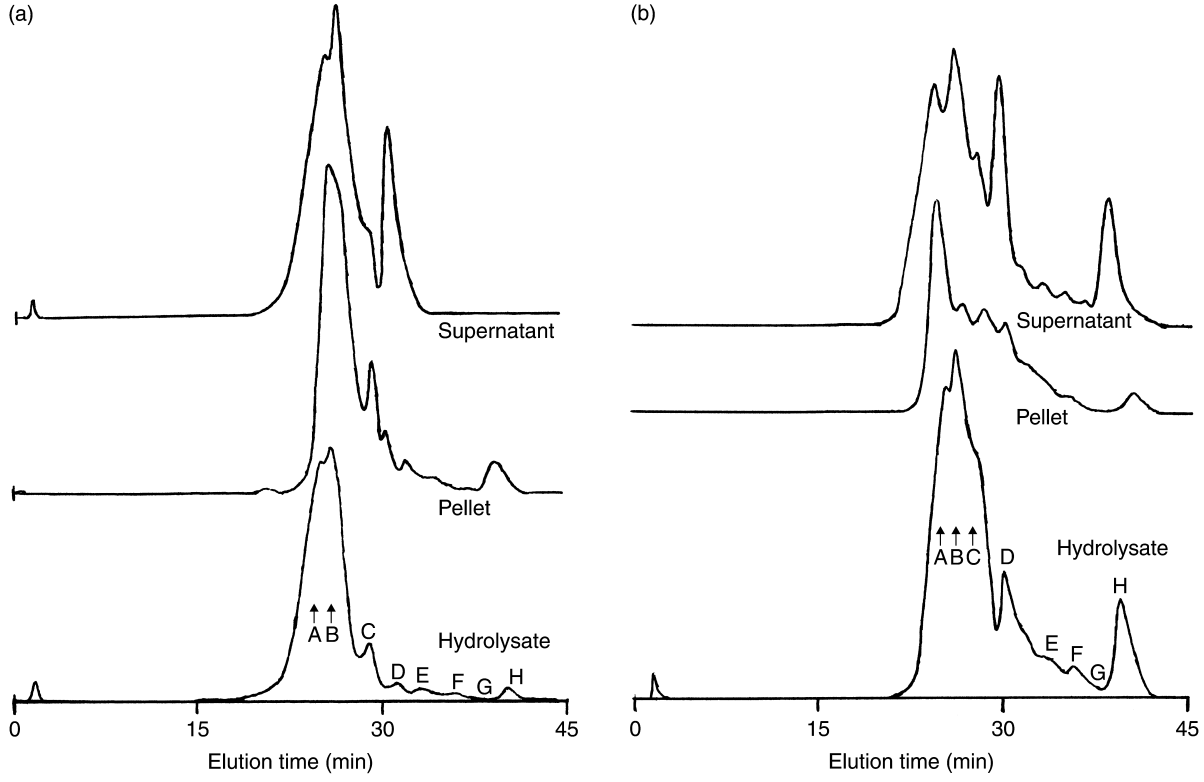

Fig. 2. Gel filtration of mycoprotein pepsin hydrolysate material and of the plastein supernatant and pellet fractions prepared from it (see text for details) on a Superose 12 FPLC column with the column eluate monitored at (a) 215 nm or (b) 280 nm.

rather selective picture and comparisons between individual runs should be treated with caution. In all cases, the ion-exchange profiles were more complex than those obtained by gel filtration, reflecting the higher resolution of the technique as well as the heterogeneity of the peptide samples. Few conclusions can be drawn except that, generally early eluting material appeared to remain largely in the supernatant fraction (the profiles of supernatant and the pepsin hydrolysate were very similar) whereas later eluting peaks were preferentially incorporated into the plastein fraction. It should be noted, however, that sample concentrations varied widely and the sensitivity range on the HPLC detector was adjusted from run to run to give overall peak height profiles of a similar size to facilitate qualitative comparisons. Thus, it is only possible in Fig. 3 to compare relative peak heights within a single run (sample) and not between runs (samples).

3.4. Amino acid composition

Table 3 shows the amino acid composition of the mycoprotein pepsin hydrolysate material and of the centrifuge supernatant and washed pellet fractions following the plastein synthesis step. From the hydrolysate data, it is clear that the mycoprotein is very hydrophilic in character. This undoubtedly is consistent with the poor plastein yields obtained in the present work, since the plastein reaction is driven kinetically by the formation of insoluble plastein material which removes the

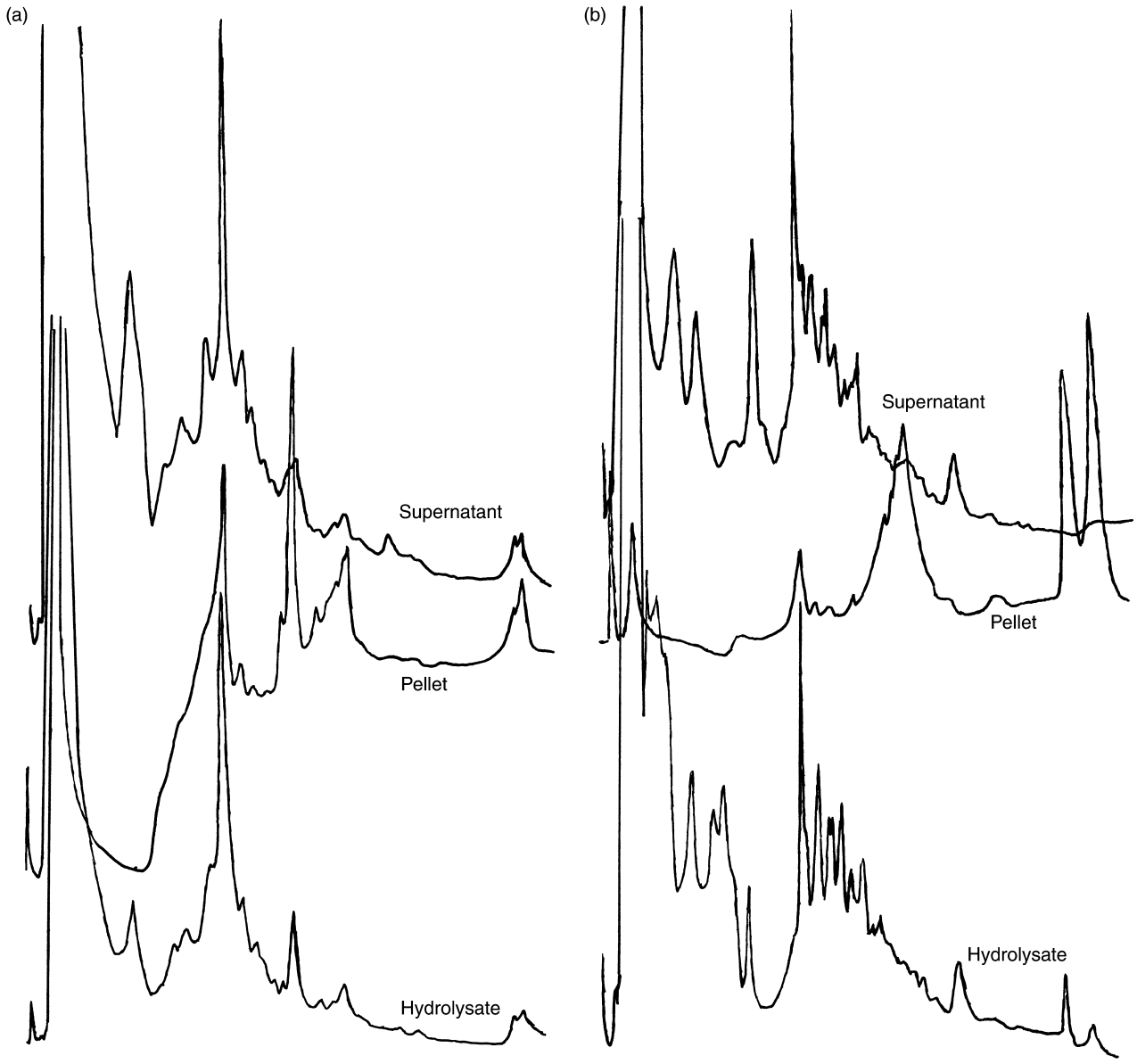

Fig. 3. Ion-exchange chromatography on a Mono Q anion-exchange FPLC column of mycoprotein pepsin hydrolysate material and of the plastein pellet and supernatant fractions prepared from it (see text for details). Column eluate was monitored at (a) 215 nm or (b) 280 nm.

Table 3
Amino acid composition (molar%) of initial pepsin mycoprotein hydrolysate and of the pellet and supernatant fractions following the plastein reaction

Amino acid	Hydrolysate	Plastein pellet	Plastein supernatant
Asp	10.60	7.72	11.30
Thr	5.58	4.72	5.91
Ser	5.92	4.20	6.29
Glu	11.10	8.98	12.30
Pro	5.22	4.17	5.15
Gly	8.14	6.22	8.88
Ala	9.56	6.60	10.20
Val	7.08	10.40	6.15
Cys/0.5 Cys	0.44	0.40	0.19
Met	1.87	2.54	1.66
Ile	5.34	9.74	4.14
Leu	8.28	15.60	6.45
Tyr	2.85	3.34	2.77
Phe	3.64	5.62	3.02
Lys	7.12	4.52	7.67
His	1.98	1.45	2.12
Arg	5.32	3.83	5.79

more hydrophobic peptides from the reaction mixture. This assertion is also shown dramatically by the data in Table 3. Taking Leu, Ile, Val and Phe as the most hydrophobic amino acids and Asp/Asn, Glu/Gln, Lys and Arg as the most hydrophilic (Kyte & Doolittle, 1982) the molar percentages of hydrophobic:hydrophilic were 24.3:34.1 in the pre-plastein hydrolysate, 41.3:25.1 in the pellet and 19.8:37.0 in the supernatant fractions after plastein synthesis. In other words, the proportion of these hydrophobic to hydrophilic amino acids changed from 0.71:1 in the initial hydrolysate to 1.65:1 in the plastein pellet and 0.53:1 in the plastein supernatant. There was thus a very marked disproportionation of hydrophobic peptides into the insoluble plastein material. While we also observed this previously in casein hydrolysates (Sukan & Andrews, 1982b) the extent of the preferential incorporation of hydrophobic material into the plastein fraction was very much more striking in this work with mycoprotein, perhaps due to the more hydrophilic nature of the overall mycoprotein hydrolysate in comparison to that from casein.

3.5. DSC

DSC gave little useful information because, probably due to the highly heterogeneous nature of the samples, no sharp, well-defined peaks were observed, indicating that there were no clearly identifiable structures with sharp melting points, but rather a large number of different but generally similar species giving broad heterogeneous peaks in the DSC profiles (Fig. 4). The trace for the mycoprotein hydrolysate material showed a single broad peak with a maximum at approximately 141°C and evidence of further minor inflection points at about 260 and 310°C, whereas the plastein pellet material gave a minor inflection at about 196°C and two broad peaks at 297 and 312°C (Fig. 4). Superficially, it would appear, therefore, that DSC was detecting structural differences

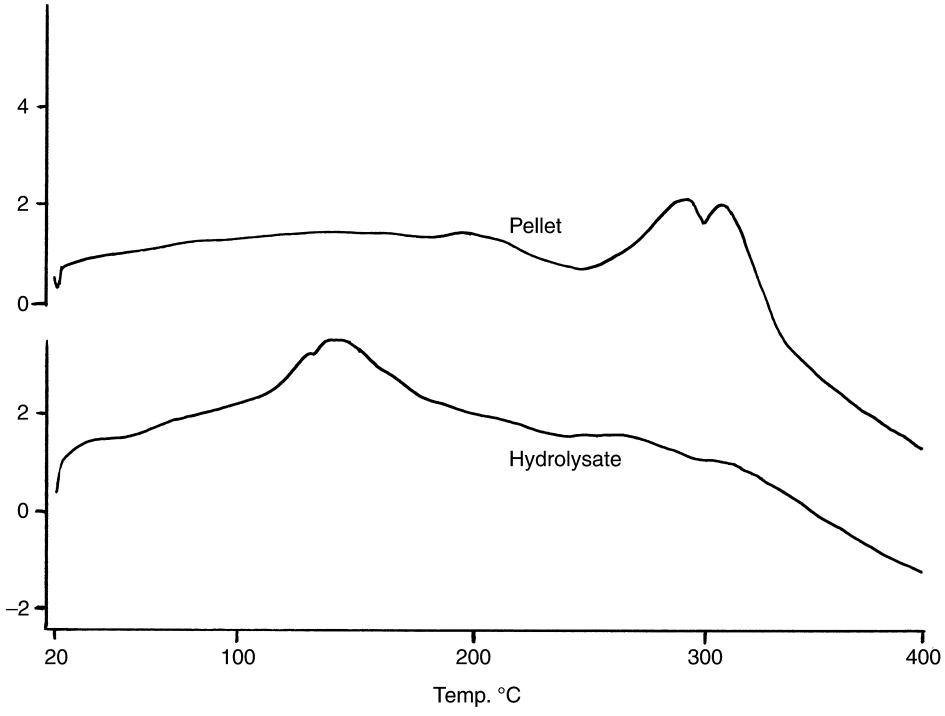

Fig. 4. Differential scanning calorimetry of mycoprotein pepsin hydrolysate material (upper trace) and insoluble plastein (lower trace) prepared from it (see text for details).

between the hydrolysate and plastein fractions, although it is difficult to conceive the formation of substantial amounts of structure that could be formed enzymically from a peptide mixture that would maintain structural integrity up to 300+°C. However, it should be noted that the yield of insoluble plastein material from mycoprotein hydrolysate was never more than 12–13% at best, so it is quite possible that the material giving a minor inflection at about 310°C in the hydrolysate material was merely that which was selectively incorporated and enriched into the plastein pellet, i.e. peptide material high in hydrophobic amino acids. This hypothesis would suggest that it is not necessary to invoke either the loss or formation of new structural elements during the plastein reaction to explain the differences seen in DSC profiles shown in Fig. 4.

4. Conclusions

It is clear that the plastein reaction can have useful, practical consequences in food applications. A number of workers have provided good evidence of the ability of the reaction to reduce bitterness in protein hydrolysates, especially soya bean, fish or milk protein hydrolysates (Fujimaki et al., 1970, 1977; von Hofsten & Lalasidis, 1976; Yamashita et al., 1970), to incorporate amino acids such as lysine, cystine or methionine into otherwise deficient hydrolysates (Eriksen & Fagerson, 1976; Fujimaki et al., 1977; Shimada et al., 1984; Yamashita et al., 1977) on the premise that they would be better absorbed than if merely mixed into diet as the free amino acids; to prepare low phenylalanine ingredients (Yamashita et al., 1976) for dietetic applications (the preferential incorporation of hydrophobic amino acids into insoluble plastein gives supernatant material reduced in these amino acids) or to prepare novel surfactant materials by incorporating fatty acid esters (Shimada et al., 1984; Watanabe et al., 1981).

The mechanism(s) by which these changes are brought about is still open to debate, however, and probably until this is resolved, it will be difficult to predict behaviour. Until then, applications are likely to be investigated on an ad hoc basis with the findings being applicable only to the particular substrate protein and set of reaction conditions studied. In early work, it was considered that the formation of insoluble plastein was merely a reversal of the normal proteinase-catalysed hydrolysis of protein, but this was based largely on work with purified small synthetic peptides, often in organic solvents, where proteinases are known to catalyse condensation reactions (Jakubke, 1987; Kullmann, 1987). Horowitz and Haurowitz (1959) were the first to suggest that condensation reactions played only a minor role and suggested that transpeptidation was the key process. This has been supported by some more recent work (Combes & Lozano, 1993; Lozano & Combes, 1991), but much of the past evidence for both the condensation and transpeptidation reactions has relied upon changes in the content of amino groups; no change being taken as evidence of transpeptidation and a decline as evidence of condensation. Unfortunately, such evidence is often unreliable. It is essential for such studies to show that the enzyme retains catalytic activity under the conditions of the plastein reaction, because low water activity, non-optimal pH, inhibition caused by high substrate or product concentration (e.g. competitive or non-competitive substrate binding), and high viscosity, can all reduce activity. It is also essential to use the same enzyme for plastein synthesis as used in the initial hydrolysis and to ensure that the initial protein hydrolysate is a limit-digest. Otherwise there will be susceptible bonds remaining in the mixture of hydrolysate peptides that can be cleaved during plastein synthesis, giving rise to the formation of new amino groups, making interpretation of reaction mechanisms difficult. Clearly, use of enzymes with different bond specificity enables hydrolysis to be a factor during the synthetic steps. Thus a constant level of amino groups may reflect merely a lack of hydrolytic activity, not a transpeptidation mechanism. Likewise, falls sometimes taken as evidence of condensation reactions (e.g. Combes & Lozano, 1993; Lozano & Combes, 1991) at high substrate concentration, may reflect either reduced diffusion in low water activity media or, more probably, a masking of peptide amino groups from the TNBS reagent by strong physical peptide–peptide interactions. The formation, simultaneously, of small amounts of high and low molecular weight material revealed by gel filtration studies is also sometimes taken as evidence of a transpeptidation mechanism, but could equally well be explained by the formation of aggregated material accompanied by some continuing hydrolysis.

The remaining possible reaction mechanism is that of a purely physical aggregation process. Aso, Yamashita, Arai and Fujimaki (1973) suggested, almost 30 years ago, that hydrophobic bonding was a major factor in soybean protein plastein formation. Although it has been suggested that the frequent definition of plastein as the formation of material insoluble in 10% trichloroacetic acid (TCA) would rule out a purely physical process, this is incorrect since addition of TCA in fact strengthens physical hydrophobic interactions between protein and peptide molecules. Since then, other workers (Edwards & Shipe, 1978; Horowitz & Haurowitz, 1959; von Hofsten & Lalasidis, 1976) have found constant amino group levels, which is just as consistent with a physical aggregation process as it is with transpeptidation, and have also concluded that physical interactions are the principal forces in plastein synthesis. Our own past work on casein (Andrews & Alichanidis, 1990; Sukan & Andrews, 1982a,b), as well as this current study on mycoprotein,

entirely supports this view. A number of reports (e.g. Arai, Yamashita, Aso & Fujimaki, 1975; Lozano & Combes, 1991) conclude that more than one mechanism may be involved, while many others have suggested a preferred mechanism but have admitted that the techniques used would not have detected small amounts of product generated by alternative pathways. The most recent elegant study of the reaction mechanisms (Stevenson, Morgan, Fenton & Moraes, 1999) used the more modern rigorous techniques of nuclear magnetic resonance and electrospray mass spectroscopy. This clearly showed that all four reaction processes referred to above (hydrolysis, condensation, transpeptidation and physical aggregation) occurred under appropriate conditions. In systems of small model peptides, transpeptidation and condensation occurred quite readily, with the latter being driven largely by the insolubility (precipitation) of the products, so hydrophobic peptides were particularly likely to take part. When high levels of hydrophobic marker peptide were added to casein hydrolysates there was also evidence of condensation reactions, particularly if exopeptidases were used as the plastein catalyst. Endopeptidases resulted in very little condensation or transpeptidation. ''Normal'' casein hydrolysates, with no added hydrophobic peptides, showed no evidence of either condensation or transpeptidation reactions. The authors concluded (Stevenson et al., 1999) that while thermodynamically, typical high substrate concentrations should lead to detectable covalent bond formation, hydrophilic peptides and some of the longer peptides present in complex hydrolysate mixtures bound to the endoproteinases and acted as inhibitors. The result was that, kinetically, any condensation or transpeptidation reactions became slow and insignificant. They therefore concluded (Stevenson et al., 1999) that physical aggregation was essentially the only significant process. With exopeptidases, the situation was less clear cut and some transpeptidation occurred.

Thus, it is now apparent that the actual mechanism may be quite complex with contributions from different pathways depending on reaction conditions, such as the nature of the substrate (i.e. peptide size and hydrophobicity), complexity of the peptide mixture, concentration, water activity, the identity of enzyme used as a catalyst and especially its endoproteinase or exoproteinase character and enzyme bond specificity. Both our earlier work on casein (Andrews & Alichanidis, 1990; Sukan & Andrews, 1982b) and this current study with mycoprotein suggest that physical aggregation processes were the most significant plastein formation reaction mechanisms. Since we used endoproteinases throughout, our findings entirely support the view of Stevenson et al. (1999) that with such enzymes and ''real life'' protein hydrolysates, this is the predominant mechanism.

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