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Application of the plastein reaction to mycoprotein: I. Plastein synthesis

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Abstract

The plastein reaction has been applied for the first time to mycoprotein following in situ digestion of crude material to solubilise protein and produce the required initial peptide mixture. Pepsin was found to be superior to the other proteinases tested (trypsin, chymotrypsin, papain, *Streptomyces griseus* proteinase) in the plastein synthesis step although in all cases yields of insoluble plastein were low (10–15%). Unusually, plastein yield was not much influenced by pH over the range 3.0–7.5, but percentage yield increased almost linearly over the peptide concentration range 11–43% (w/w) while the absolute yield of plastein increased exponentially over this range. Incubation at different temperatures showed that the rate of plastein formation was highest at 65°C, reaching a maximum in 4–5 h but, although not reaching maximum yield for 24 h, a temperature of 37°C gave approximately 25% greater yield. In general terms, the results suggested that mycoprotein peptides represented a rather poor substrate for plastein synthesis and only opaque, viscous solutions were obtained rather than the more common thixotropic gel structures.

Keywords: Quorn; Mycoprotein; Plastein synthesis; Enzymic modification

1. Introduction

Although the plastein reaction was first described almost 100 years ago, it is still relatively unknown and under-exploited in practical terms. In fact it remains at the stage of an intellectual curiosity and there is continuing argument about the precise reaction mechanism (or mechanisms, for recent work suggests that several pathways may play a role). Earliest thoughts were that it was simply a kinetically-driven reversal of the usual hydrolysis of proteins to peptides (working with high peptide concentrations) in which a proteinase catalyses a condensation reaction process, resulting in "new" protein material of high molecular weight (Determan, Eggenschwiler & Michel, 1965; Fujimaki, Kato, Arai & Yamashita, 1971; Yamashita, Arai, Tanimoto & Fujimaki, 1973). Other workers (Combes & Lozano, 1993; Fujimaki, Arai & Yamashita, 1971; Lozano & Combes, 1991) have suggested that transpeptidation is the prime mechanism, but there are also reports (Edwards & Shipe, 1978; von Hofsten & Lalasidis, 1976) that few new covalent bonds are formed and that physical forces, such as hydrophobic and ionic bonds, are the most important — a view strongly supported by our own past work (Andrews & Alichanidis, 1990; Sukan & Andrews, 1982a).

Although uncertainty remains, therefore, over the precise reaction mechanism, it has been shown unequivocally that there is a requirement for an active proteinase to catalyse the reaction (Andrews & Alichanidis, 1990), despite the fact that the optimum pH of plastein formation may be very different from that of the same enzyme acting in the usual manner as a hydrolase. This was supported by the findings (Sukan & Andrews, 1982a) that both the rate of formation and the yield of plastein increased with rising temperature up to about 50°C but, at 70°C, although the initial rate was very rapid, the reaction soon stopped and overall yield was much lower than at 20–50°C. This is consistent with the denaturation and heat inactivation of pepsin, which was the enzyme used as the catalyst.

In practical terms, whatever the mechanism, the plastein reaction results in the conversion of freely soluble concentrated solutions of peptides into reaction mixtures containing a large amount of material that is insoluble

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in aqueous buffers, ethanol or acetone. The most striking change is in physical properties, with a marked increase in viscosity, often leading to the formation of thixotropic gels. There is also usually an obvious visual change and clear, transparent peptide solutions become progressively more cloudy and opaque. Another curious but potentially valuable attribute of the plastein reaction is its effect on bitterness, and since the earliest reports (Yamashita, Arai, Matsuyama, Kato & Fujimaki, 1970), there have been many studies showing that peptide-containing hydrolysates of soya bean, fish, caseins and many other proteins, that often have marked bitterness, become much less bitter or lose bitterness altogether when subjected to the plastein reaction. It is probable that this loss of bitterness is related to the preferential involvement of hydrophobic peptides in plastein formation (Aso, Yamashita, Arai & Fujimaki, 1973; Sukan & Andrews, 1982b), as it is precisely these peptides which are most strongly implicated in generating bitterness.

It is these practical benefits of the plastein reaction which make it of potential interest in food applications, as it provides an attractive route to the production of highly nutritious, low-calorie thickening and gelling agents for use in novel food products. Although it remains to be tested, it may also be valuable in reducing allergenicity, as it is well known that protein hydrolysates are usually much less allergenic than native proteins. The reaction may also be able to detoxify toxic proteins such as legume lectins and trypsin inhibitors as an alternative to detoxification by heat treatments (denaturation). The ability of the plastein reaction to impart textural structure to simple hydrolysates could have useful dietetic applications, especially in clinical patients with impaired digestive processes.

Providing that economic factors relating to the application of the plastein reaction to food materials are acceptable, it could be applied to almost any food protein and requires only "food-acceptable" enzymes as catalysts, operating without the need for any undesirable solvents or reagents.

As part of an ongoing but intermittent study of the plastein reaction, in this paper we report its application to a mycoprotein material (Quorn) produced by the fermentation of a strain of *Fusarium graminearum* (Rodger & Angold, 1991; Trinci, 1994) and frequently used in food products as a meat-analogue, following a heat-treatment step to reduce the nucleic acid (RNA) content (Anderson & Solomons, 1984; Maul, Sinskey & Tannenbaum, 1970). This is a relatively high-cost food protein, so may not be an ideal candidate for further manipulation via the plastein reaction, but our study does extend the reaction to a new class of food proteins that have not been studied in this context before and shows the universal applicability of the reaction to all proteins.

2. Materials and methods

Mycoprotein samples (Quorn^{TM}) were very kindly supplied by Marlow Foods Ltd. Stokesley, UK. They were in the form of moist pressed "cakes" of *F. graminearum* fungal hyphae, each cake having dimensions of approximately 90×60×8 mm and weighing approximately 40 g. They had been processed (Rodger & Angold, 1991) by Marlow Foods Ltd. by harvesting the filamentous fungus grown on a starch based medium and heating at 65°C for 10 min to break down nucleic acids (predominantly RNA). The heated material was filtered and centrifuged briefly (10–20 s) at 90°C in a basket centrifuge and nucleic acid fragments removed in the filtrate and centrifuge liquor. This process reduced the RNA content from a level of about 8%, which is unacceptably high for food applications, to less than 1% on a dry weight basis. WHO/FAO recommendations limit the dietary intake of RNA to 2 g per day from novel food sources. The mycoprotein "cakes" represented material retained in the basket centrifuge and had not been processed further. They were stored at −20°C until required.

Mycoprotein cakes were analysed for moisture content by heating finely chopped and weighed samples at 105°C for 3 h and reweighing. Heating for a further 3 h in a muffle furnace at 600°C, cooling and reweighing, gave the ash content. Protein content was determined as Kjeldahl nitrogen times 6.25. Total fat was determined by solvent extraction using petroleum ether in a Soxhlet apparatus, followed by solvent removal and weighing.

Trypsin, TLCK-treated (EC3.4.21.4), pepsin 1:60 000 (EC3.4.23.1), Protease Type XIV from *Streptomyces griseus* (Pronase; E.C.3.4.21.4), ∝ — chymotrypsin Type VII TLCK — treated (EC3.4.21.1) and papain 2× crystallised (EC3.4.22.2) were all from Sigma Ltd. Poole, UK, as were sodium dodecyl sulphate (SDS) and trinitrobenzene sulphonic acid (TNBS). All other reagents were Analytical grade from Merck.

For small-scale extraction of protein or peptides from the mycoprotein cake, 40 g of the cake material was suspended in water, to a volume of 100 ml, and blended into a fine slurry with an Ultra Turrax blender. Portions of 5 g of the slurry were mixed with 5 ml H_2O; 5 ml of 0.1 M sodium phosphate, pH 7.0, buffer, 5 ml of this pH 7.0 buffer containing 1% SDS, 5 ml 0.1 M sodium phosphate, pH 8.0, buffer containing either 1.5 mg trypsin or 5.0 mg Pronase, or 5.0 ml 0.1 M formic acid adjusted to pH 1.8–2.0 with 1 M HCl and containing either 1.0, 10.0 or 50.0 mg of pepsin. All samples were then incubated at 37°C for 4 h and then filtered. Nitrogen content of the filtrates was measured by the Kjeldahl procedure and multiplied by 6.25 to give the protein contents.

For enzymic release of peptides from the crude mycoprotein cake material by proteinases, 5 g portions

of the mycoprotein slurry were mixed, either with 1.0 ml of 1 M formic acid and adjustment of the pH to 2.0 with 1 M HCl and volume to 10 ml with H_2O followed by the addition of 2.5 mg, or 10 mg of pepsin, or with 5 ml 0.1 M sodium phosphate buffer, pH 8.0 (giving a final pH of 7.5), and 2.5 mg of trypsin or 2.5 mg of Pronase. Approximately 1 mg/ml NaN_3 was added as preservative and all samples incubated at 37°C. Portions of 1.5 ml were withdrawn from each mixture after 0, 1, 2, 4, 8, 24 and 72 h, centrifuged at 12000 ***g*** for 10 min, and the amount of peptide material solubilised into the supernatant fractions determined by measurement of amino groups with TNBS. For this, 10 μl samples of supernatant were mixed with 1.0 ml of 0.1 M sodium borate buffer, pH 9.4, 1.0 ml of 10% (w/v) SDS and 1.0 ml 0.1% (w/v) TNBS. The mixtures were incubated at 37°C for 60 min, 1.0 ml 0.5 M HCl was added and optical density recorded at 410 nm.

On the basis of these results, a larger-scale pepsin digestion was undertaken to provide peptide material for use in all subsequent experiments on plastein synthesis and properties. For this, 500 g of mycoprotein cake was mixed with 1200 ml H_2O and homogenised first with a domestic blender and then with the Ultra Turrax. Formic acid (60 ml) was added, taking the pH to 1.6, followed by 0.15 g NaN_3 as preservative and then 5.0 g pepsin. The mixture was incubated at 37°C for 48 h and centrifuged at 3000 ***g*** for 15 min, giving 1500 ml of supernatant, pH 1.9, which was divided into subsamples and stored at −20°C and lyophilised as required.

For plastein synthesis, in a typical experiment, 8 g of lyophilised Quorn peptide mixture was mixed with 12.0 ml H_2O and 4.0 ml of 0.5 M sodium acetate buffer, pH 5.0, containing 20 mg/ml of pepsin were added. This gave an enzyme to substrate ratio of 1:100 and a peptide concentration of 33% by weight. In order to study the influence of temperature on plastein formation, this mixture was divided into 20 preweighed Eppendorf tubes, each containing 1.0 ml, and separate tubes were incubated for various times at 20, 37, 50 or 65°C. After incubation, the tubes were centrifuged for 10 min at 13000 rpm in a microcentrifuge, supernatants decanted off and discarded and the plastein pellets washed once by resuspension and recentrifugation with 1.0 ml 0.1 M sodium acetate pH 5.0 buffer and once with 1.0 ml H_2O, after which the pellets were dried for several days over P_2O_5 and NaOH pellets to constant weight. A similar approach was used to study the influence of other proteinases (substituted for pepsin in the above), pH (replacing the 0.5 M acetate pH 5.0 in the above with 0.25 M KH_2PO_4 adjusted to the required pH with 1.0 M HCl or NaOH) and peptide concentration (by adding appropriate weights of solid peptide, H_2O and buffer to individual tubes). Unless stated otherwise, an incubation time of 42 h at 37°C was used in the plastein synthesis step.

3. Results and discussion

3.1. Preparation of peptide starting material

The fungal hyphal wall consists largely of chitin and β-glucans, which contribute to the high non-digestible fibre content. The gross composition of the mycoprotein cake material is shown in Table 1. McCance and Widdowson (1991) quote a moisture content of 75% and a protein value which on a dry weight basis, would be 47.2%. Thus with the exception of lipid content, where we obtained an unexpectedly low reading, our values for moisture, ash and protein are in good agreement with literature reports. Most importantly, in the context of this study, it is clear that protein constitutes about half of the dry weight or between 11.8 and 15% of the moist mycoprotein cake.

Being a whole tissue material, the crude protein content will of course be made up of many different proteins which will differ substantially in terms of both composition and heat stability. The heating step at 65°C is rather similar to that used for typical food pasteurisation processes and there are many proteins that will survive it unscathed, while the centrifugation at 90°C, which is likely to cause much more extensive protein denaturation, is of short and rather imprecise duration, so it is difficult to know what proportion of protein would have been denatured. It is possible that small amounts of native soluble proteins would have been lost in the filtrate or centrifuge liquor but much of the protein is likely to be denatured and insoluble. This was confirmed by initial attempts to solubilise the protein from mycoprotein cake homogenates (Table 2), from which it could be seen that simple water or buffer extraction solubilised only a small proportion of the protein present and even the addition of the powerful anionic detergent sodium dodecyl sulphate (SDS) brought about little improvement. It thus seems likely that 10–15% of the protein remains in a soluble form while the rest is denatured and insolubilised.

Table 1
The composition of quorn mycoprotein

	This study (%)	Rodger and Angold (%)	McCance and Widdowson (%)
Moisture	74.1	70	75
Dry weight (%)			
Protein	53.8	50	47.2
Ash	3.1	3.3	N.D.
Lipid	1.2	12.4	14.0
RNA	N.D.	1.1	N.D.
H_2O soluble sugars	N.D.	2.7	8.0
Fibre	N.D.	24.6	

Table 2
Solubilisation of quorn mycoprotein

Extracting medium[a]	Protein extracted (%)[b]
H_2O	14
0.1 M Na phosphate pH 7.0	12
0.1 M Na phosphate pH 7.0 + 1% SDS	14
Trypsin (1.5 mg)	53
Pronase (5.0 mg)	90
Pepsin (1.0 mg)	78
Pepsin (10.0 mg)	91
Pepsin (50.mg)	93

[a] See text for details.
[b] Based on 13% protein moist weight; soluble protein determined by Kjeldahl N values.

Fortunately the requirements for starting material for the plastein reaction are that there should be a high concentration of relatively small peptides and there is no need for intact protein molecules. Denatured proteins can thus provide an ideal source as they are often more readily hydrolysed than native globular protein structures. We therefore applied an initial in situ digestive step directly to mycoprotein cake material and, as can be seen (Table 2), addition of proteinases greatly facilitated the solubilisation of proteinaceous material. The highly non-specific proteinase, Pronase (which is in fact a mixture of several different *S. griseus* enzymes), rapidly solubilised nearly all of the protein and was rather more effective than the much more specific trypsin. Both of these enzymes work best at pH values close to neutrality or slightly basic. Pepsin, which functions optimally at pH values close to 2.0, where the extreme pH often contributes to substrate protein unfolding, and hence the accessibility of susceptible peptide bonds, appeared to be as effective as Pronase. Table 2 lists the amounts of protein solubilised from the mycoprotein cake, as determined directly by Kjeldahl nitrogen analysis. In general terms results showed that the actual amounts of protein solubilised by the three enzymes were not very different. When this same experiment was repeated using the TNBS reaction to measure the concentration of free NH_2 groups in the soluble fraction, the time course for the hydrolysis reactions (Fig. 1) showed very clearly that the non-specific Pronase generated far more NH_2 groups than the more specific proteinases trypsin and pepsin, which were broadly similar to each other. Increasing the proportion of pepsin had only a small effect, showing that, during the course of the experiment, this enzyme had essentially produced a "limit digest" where all, or most of, the potentially susceptible peptide bonds had been hydrolysed. After a 24 h digestion period, Pronase had generated 4–5 times more NH_2 groups than trypsin or pepsin, which was consistent with the much more non-specific behaviour of this enzyme. When coupled with the Kjeldahl nitrogen figures, this showed that Pronase gave hydrolysates with

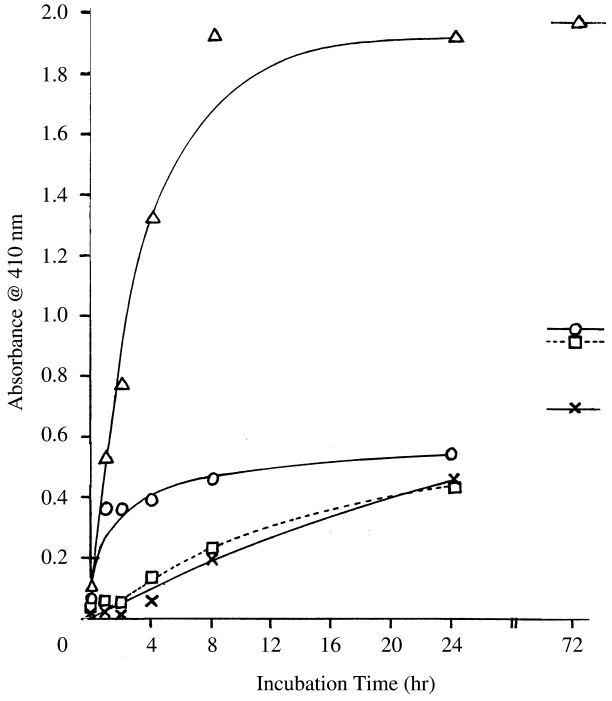

Fig. 1. The hydrolysis of mycoprotein by proteinases as measured by amino group formation (TNBS) assay. Samples of mycoprotein in pH 2 buffer for pepsin or pH 8 buffer for trypsin and Pronase were incubated at 37°C with 0.25 mg/ml of pepsin (×), 1.25 mg/ml pepsin (□), 0.25 mg/ml trypsin (○) and 0.25 mg/ml Pronase (△).

a much smaller average peptide size than the other enzymes. Without knowing the composition, proportions and molecular weights of the consitutents of the mycoprotein fraction, it is not possible to be precise about average peptide size, but assuming on the basis of our work with other proteins (e.g. Andrews & Alichanidis, 1990; Sukan & Andrews, 1982a,b) that the average molecular weight of peptides in pepsin limit digests is 800–1200, it is probable that Pronase digestion of mycoprotein would have generated peptides with molecular weights of 200–300, i.e. on average di- and tripeptides. These are likely to be too small for successful plastein production, so for subsequent work we concentrated on pepsin as the enzyme for use in preparing peptide material for plastein synthesis.

3.2. Influence of proteinase identity for plastein synthesis

Identical portions from a common batch of mycoprotein pepsin peptides were used as starting material for plastein synthesis catalysed by different proteinases and it was found (Table 3) that a greater weight of insoluble plastein was formed when pepsin was employed for the synthesis step than when other proteinases were used. Although differences in yield were relatively small, the results did support previous work (Sukan & Andrews, 1982a) in which it was clearly shown to be

Table 3
The influence of proteinase identity on mycoprotein plastein synthesis

Enzyme	Plastein pellet weight (mg)[a]	Plastein yield (%)[b]
Pepsin	38.6	13.0
Trypsin	37.5	12.6
Chymotrypsin	30.9	10.4
Papain	29.9	10.0
Control (no enzyme)	1.9	0.6

[a] Reaction performed at pH 5.0 and E:S ratio of 1:100.
[b] Each sample vial contained an initial 298 mg of mycoprotein peptides.

beneficial to use the same enzyme with the same peptide bond specificity for both the protein hydrolysis step to generate the initial peptide mixture and for the plastein synthesis step. This is because there is always potential for further hydrolysis to occur at the same time as the synthesis reaction, but if most susceptible bonds have already been cleaved during hydrolysis this will be a less important factor than when an enzyme with a different bond specificity is used.

It should be noted, however, that even under the most favourable situation where pepsin was used, the dry weight of plastein formed (38.6 mg, Table 3) corresponded to only 13% of the dry weight of the initial peptide mixture taken. This was approximately half the yield reported previously using a range of milk proteins (Sukan & Andrews, 1982a). In accordance with this it was found that application here of the plastein reaction to mycoprotein peptide mixtures led to plastein products that took the physical form of viscous thixotropic solutions rather than the thixotropic gels observed with milk and most other proteins. Since plastein formation preferentially involves the more hydrophobic peptides in a mixture, this probably indicates the unusual hydrophilic nature of the mycoprotein derived peptides (Brownsell, Williams & Andrews, 2001).

3.3. Influence of pH on plastein synthesis

In virtually all of the literature reports (e.g. Edwards & Shipe, 1978; Fujimaki et al., 1971, 1977; Lozano & Combes, 1991; Sukan & Andrews, 1982a) giving details of the conditions required for plastein synthesis, there is a clear optimum pH value, generally in the region pH 5.0–7.0, but Fig. 2 shows no particular optimum over the whole pH range studied (pH 3.0–7.5). In fact, the plastein yields obtained with pepsin used as catalyst, for both the initial hydrolysis and plastein synthesis stages, fell on a straight line almost parallel to the pH axis, rising by only a small percentage as pH increased. The reasons for this lack of a pH optimum are unknown, but it may reflect a dominant role for hydrophobic interactions which are largely independent of pH in the plastein synthetic mechanism rather than chemical

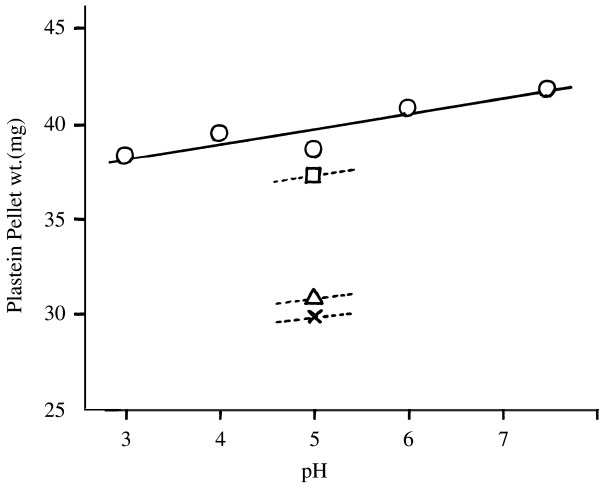

Fig. 2. Influence of pH on plastein formation. Portions of mycoprotein peptide were dissolved in 0.25 M potassium buffer of the required pH as a 33% (w/v) solution and incubated at 37°C for 24 h with proteinase (E:S = 1:100), to generate material (see text for details). Plastein formed with pepsin catalyst (○) or at pH 5 only, with trypsin (□), chymotrypsin (△) or pepsin (×).

reactions, such as transpeptidation and isopeptide bond formation or other physical forces such as electrostatic and hydrogen bonding, all of which are influenced by pH and have been implicated in mechanisms suggested in previous reports with different materials. The mycoprotein peptides used here are perhaps atypically hydrophobic and a dominant hydrophobic mechanism would be consistent with the low plastein yields obtained, their physical properties and the unusually extreme partitioning of hydrophobic peptides into the plastein pellet (Brownsell et al., 2001).

3.4. Influence of peptide concentration on plastein synthesis

When plastein yield was plotted versus the concentration of mycoprotein peptides in the synthesis mixture, a curved plot, almost exponential in shape, was obtained (Fig. 3). If the same data are plotted as percentage yield (i.e. percentage of the peptides recovered in the plastein pellet), the data best approximate to a straight line plot passing through the origin. Again, these findings were most unusual because other literature reports (Fujimaki et al., 1977; Lozano & Combes, 1991; Sukan & Andrews, 1982a) with different starting materials show that plastein formation is at a maximum when peptide concentrations are in the region of about 20–40% by weight and falls off sharply both above and below that range. Thus convex plots for both curves were expected, especially when percentage yield was plotted. Our results were consistent with an entropy-driven reaction mechanism involving hydrophobic interactions rather than the more common kinetically-driven one.

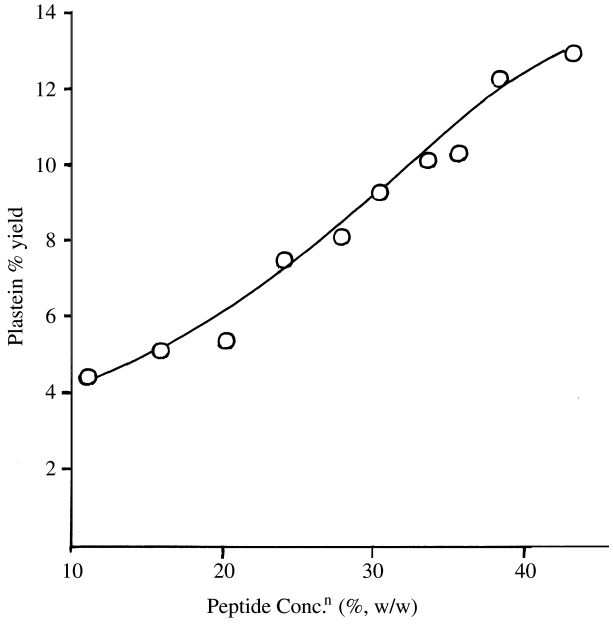

Fig. 3. Influence of mycoprotein peptide concentration on plastein yield using pepsin at pH 5.0 and E:S = 1:100 as catalyst.

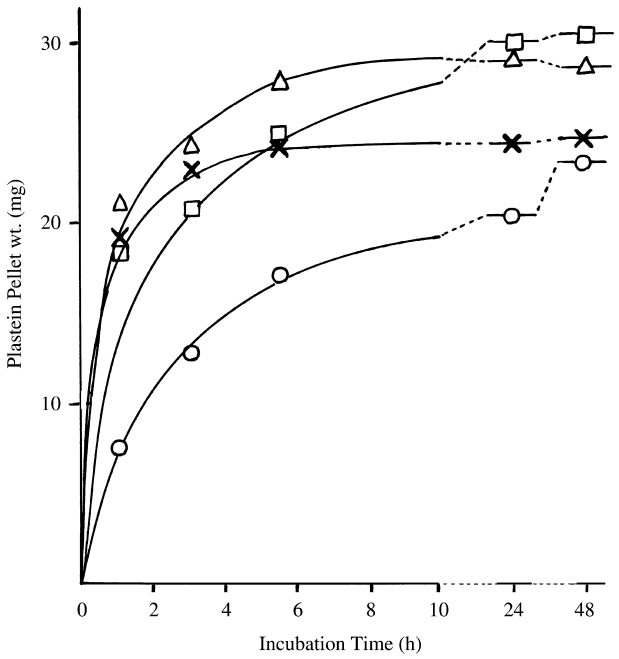

Fig. 4. Influence of incubation temperature on the yield and rate of formation of insoluble plastein from mycoprotein hydrolysate material using pepsin at an enzyme:substrate (E:S) ratio of 1:100 at pH 5.0. 20°C (○); 37°C (□); 50°C (△) 65°C (×).

3.5. Influence of temperature on plastein synthesis

Incubation of samples of mycoprotein peptides with pepsin at various different temperatures led to the results shown in Fig. 4. It can be seen that at the highest temperature studied (65°C) plastein formation was rapid, with 93% of the maximum amount being generated within 3 h. The maximum was reached within 6 h but thereafter there was no further plastein formation. The maximum yield attained, however, was substantially lower than with incubations at 37C or 50°C, which probably indicates a relatively rapid denaturation and inactivation of the pepsin catalyst.

Incubation at 50°C (Fig. 4) led to a slightly slower rate of plastein formation in percentage terms (83% of maximum after 3 h; 95% after 6 h), although absolute amounts obtained were higher than at 65°C, and the maximum was reached within 24 h. At 37°C plastein formation was slower still (70% of maximum in 3 h; 82% in 6 h) but, after 24 h, had attained a level 3% above the maximum achieved at 50°C. Further incubation at 37°C led to a very small further increase in yield (about 1%), which is probably not significant. As expected, incubation at room temperature (approx. 20°C) gave the slowest rate of plastein formation but it is worth noting that, after 48 h, the plastein yield had almost reached the levels obtained at 65°C and, had incubation been prolonged further, might have exceeded it.

These findings are entirely consistent with literature trends for other systems and agree well with our earlier work on milk protein hydrolysates (Sukan & Andrews, 1982a). Thus, at least when pepsin is employed as the catalyst, the optimum temperature range is 37–50°C. Since hydrophobic forces are stronger in aqueous solutions as temperature rises, the plastein yield profile might be similar (even if they were the sole driving forces) but the results are also entirely consistent with enzyme-catalysed kinetic mechanisms and little plastein was formed in controls with no added pepsin, so probably both mechanisms are involved.

4. Conclusions

Peptides derived from a mycoprotein source provide relatively poor substrates for plastein formation, giving rise to opaque, thixotropic, viscous solutions rather than the gelled products generally given by other food proteins. This physical behaviour is likely to be linked to the rather poor yields of insoluble plastein material formed. As in other studies, it is clear that it is preferable to use for the plastein synthesis stage the same proteolytic enzyme as used in the initial protein hydrolysis step and both this and the influence of reaction temperature on plastein synthesis suggest that, as in all other reports, there is a requirement for an active proteinase to catalyse the synthetic step. However, major deviations from other work are seen in the experiments in which plastein formation was measured at different pH values and at different peptide concentrations. The results in this study suggested that hydrophobic interactions played an unusually important part in the synthesis mechanism

and this was strongly supported by analytical data given in the accompanying paper (Brownsell et al., 2001).

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