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The Plastein Reaction Revisited: Evidence for a Purely Aggregation Reaction Mechanism

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ABSTRACT

Various aspects of the plastein synthesis reaction were investigated using peptides derived from casein as substrate. With peptides obtained by partial acid hydrolysis a clear requirement for a proteinase to catalyse plastein synthesis was demonstrated and, although enzymes whose hydrolytic activity had been inhibited may act as rather inefficient catalysts, the native active enzymes were preferred. Blockage of peptide NH_2 or COOH groups reduced plastein yield but did not prevent synthesis. Results following addition of water-miscible organic solvents to reaction mixtures were more consistent with increased solubility of hydrophobic amino acids and peptides rather than with the influence of viscosity changes. Although plastein separates out of the reaction mixture in the form of a gel or precipitate and can be collected as an insoluble centrifuge pellet, on repeated washing and incubation it was gradually solubilised even in aqueous buffers, showing clearly that plastein formation is a reversible process. This was in effect confirmed by ion-exchange chromatography and gel filtration experiments, in which there were some quantitative differences in peptide composition between peptide hydrolysate starting materials and resolubilised plastein pellets produced from them but no qualitative differences. This showed clearly that no appreciable amounts of new peptides were formed and ruled out covalent bond formation in a reversed hydrolysis or a transpeptidation pathway as the reaction mechanism. This conclusion was confirmed by SDS-PAGE and by preliminary small-angle neutron scattering experiments. We therefore conclude that the plastein synthesis reaction is a purely entropy-driven physical aggregation process.

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INTRODUCTION

The plastein reaction has a history going back for over 80 years but, with the exception of the work of Fujimaki's group (e.g. Fujimaki *et al.*, 1977), studies of it have been infrequent and rather fragmented. Perhaps because of this the mechanism of plastein formation has never been satisfactorily established and is still open to debate. A number of potential applications of the plastein reaction have been suggested (Eriksen & Fagerson, 1976; Fujimaki *et al.*, 1977) but few if any of them show economic advantages at present over competing processes, with the net result that the reaction remains largely an academic curiosity.

The plastein products form thixotropic gels or thixotropic viscous solutions, depending on concentration, which are stable over a very wide pH range (e.g. Fujimaki *et al.*, 1971, 1977; Eriksen & Fagerson, 1976; Fruton, 1982; Sukan & Andrews, 1982*a*,*b*; Jakubke, 1987; Kullmann, 1987) and quite a broad temperature range, from at least 0 to 70°C (Sukan & Andrews, 1982*a*,*b*). These physical properties could be useful in the food industry, where the reaction might find a role in the preparation of thickening or gelling agents in products such as novel desserts, for example. They would have the merits of being nutritious, low in calorific value, being an almost odourless, colourless and bland base suitable for the addition of a variety of flavours, etc., and of being 'natural'. In order to realise these benefits, however, it is essential to improve the economics of plastein formation very considerably and in order to do this it is, in turn, necessary to understand more about the reaction mechanism.

Originally it was thought (e.g. Fujimaki *et al.*, 1971) that the reaction was simply a reversal of the usual hydrolysis of proteins by proteinases to generate new proteins or high molecular weight polypeptides from a starting material of small peptides and amino acids. If this were the case, there would be close parallels with the well-established proteinase-catalysed condensation of protected amino acid derivatives to small peptides at very much lower reactant concentrations and sometimes in media containing additions of organic solvent (Fruton, 1982; Jakubke, 1987; Kullmann, 1987). Any apparent similarities do not bear close scrutiny, however, because the latter works best with amino acid derivatives and is not satisfactory with peptides larger than about a tetrapeptide because of competing hydrolysis reactions (Kullmann, 1987), has a pH optimum similar to that of the optimum for the hydrolysis reaction with the enzyme in question, gives insoluble but not aggregated products, is dependent on proteinase specificity, and is a true condensation involving a loss of free amino and carboxyl groups. None of these appears to hold for the plastein reaction, which is independent of proteinase specificity and hydrolysis pH optimum (always about 5 for the

plastein reaction regardless of the proteinase used), works best with peptides (mol. wt about 500–1000 is preferred) which do not require prior modification or protection, gives insoluble but highly aggregated products which under non-denaturing conditions appear to have high molecular weight, and does not involve any significant loss in NH_2 or carboxyl groups (Horowitz & Haurowitz, 1959; von Hofsten & Lalasidis, 1976; Sukan & Andrews, 1982*a*,*b*).

Some reports (e.g. Fujimaki *et al.*, 1977) have suggested that the key mechanism in plastein formation is transpeptidation, hydrophobic bonding or ionic bonding, or perhaps a combination of factors including these and covalent bond formation. Other workers (von Hofsten & Lalasidis, 1976; Edwards & Shipe, 1978) reported little if any formation of new peptide bonds and our past work (Sukan & Andrews, 1982*a*,*b*) supported this view. Indeed, our results were not consistent with either a transpeptidation mechanism or a major role for ionic bonding and we concluded that the primary mechanism was via hydrophobic interactions with only a minor part being played by ionic bonding and negligible covalent bond formation. The present report gives the results of further experiments aimed at describing plastein properties and giving further information on the formation mechanism.

MATERIALS AND METHODS

Materials

Porcine pepsin (EC 3.4.23.1) 1:60 000, α-chymotrypsin (EC 3.4.21.1) Type VII TLCK-treated, pepstatin A, ovalbumin Grade VI, bovine serum albumin (product No. A7638), β-glucosidase (EC 3.2.1.21, product No. G0395) and phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. Ltd (Poole, Dorset, UK). Papain (EC 3.4.22.2) and horseradish peroxidase Grade 1 (EC 1.11.1.7) were from Boehringer Mannheim (BCL, Lewes, Sussex, UK). All other reagents were from British Drug Houses Ltd (Poole, Dorset, UK). Sephadex and FPLC apparatus and columns were from Pharmacia–LKB Ltd (Milton Keynes, UK).

Methods

Enzymic hydrolysis of sodium caseinate was achieved by incubating 5% (w/v) solutions, adjusted to pH 1·8 with formic acid for pepsin digestion or to pH 7·0 with NH_4HCO_3 for papain and chymotrypsin treatments, with proteinase added at an enzyme:substrate ratio of 1:100 (w/w) for 48 h at 37°C followed by lyophilisation.

Acid hydrolysis was performed by mixing 20 g of sodium caseinate with 50 ml H_2O and 10 ml conc. H_2SO_4 and heating at 80°C for 2·5 h and filtering out any residual insoluble material. Barium carbonate was then added carefully to the filtrate to the point at which no more effervescence of CO_2 or precipitation of $BaSO_4$ occurred. The mixture was then centrifuged at 1000*g* for 20 min and the supernatant peptide solution filtered to remove any remaining particles of precipitate and lyophilised. These acid hydrolysis conditions were found by assay of amino groups (Sukan & Andrews, 1982*a*) to give about the same extent of hydrolysis (average peptide size) as the above treatment with pepsin.

For plastein formation, unless stated otherwise, peptides were made up as 30–35% (w/v) solutions in 0·1M sodium acetate buffer (pH 4·8) containing 10 mM sodium azide and incubated at 37°C for at least 24 h. When plastein yield was to be measured, the sample was stirred up with 10 volumes of 0·1M sodium phosphate buffer (pH 7·0), the mixture centrifuged at 3000*g* for 20 min, supernatant decanted off as completely as possible and the plastein pellet dried overnight at 80–90°C, cooled and weighed.

TABLE 1
Requirement for Active Proteinase in Plastein Formation

Catalyst	*Visual observations*	*Plastein pellet weight (mg)*
None (control)	No turbidity or precipitate	0
Pepsin	Thixotropic gel	62·0
Pepsin (inactivated at pH 12, 45 min)	Slight turbidity, no gel formation	7·1
Pepsin (heat inactivated, 80°C, 10 min)	Some turbidity, no gel formation	8·0
Pepsin + pepstatin (50-fold molar excess)	Some precipitate formation, no gel	8·2
Papain	Turbid thixotropic viscous liquid	24·0
Papain + ammonium persulphate[a]	Some precipitate immediately on persulphate addition, no apparent increase during incubation	6·1
Papain + iodoacetic acid[a]	As above	2·5
Chymotrypsin	Thixotropic gel	35·2
Chymotrypsin (PMSF-treated)	No turbidity or precipitate	<0·5
Ovalbumin	Slight precipitation (denatured ovalbumin?)	<0·2
Bovine serum albumin	No turbidity or precipitate	0
β-Glucosidase	Trace precipitate	0
Horse-radish peroxidase	No turbidity or precipitate	0

[a] Five milligrams of ammonium persulphate or iodoacetic acid was added to the 0·1 ml of papain solution immediately before peptide addition.

In experiments using peptides obtained by acid hydrolysis, the lyophilised peptides were made up as a 46% (4·0 g dissolved in a final volume of 8·5 ml) solution in 0·5M sodium acetate buffer (pH 4·7) and 0·7 ml portions mixed with 0·1 ml samples of aqueous solutions of the various proteins and enzymes (30 mg/ml), shown in Table 1, to give approximately 40% (w/v) solutions of peptide containing peptide:catalyst (enzyme, inhibitor, protein, etc.) ratios of 100:1.

Dabsyl labelling (Tzeng, 1983) of peptides was achieved by treating a solution of peptide (20 mg/ml) in 0·2M sodium borate buffer (pH 9) with an equal volume of 10 mM 4-dimethylamino-azobenzene-4′-sulphonyl chloride (dabsyl chloride) in acetone. The mixture was warmed at 60°C for 15 min, filtered and the filtrate applied to a gel filtration column of G-25 Sephadex fine grade made up in 0·2M ammonium hydrogen carbonate. The orange-coloured zone of labelled peptides was easily separated from the excess reagent and was collected and lyophilised.

For chemical modification with various acid anhydrides, portions (1·0 ml) of pepsin-produced casein peptides (460 mg/ml) in 0·2M sodium phosphate buffer (pH 7·5) were mixed with acetic anhydride (120 μl, butyric anhydride (180 μl) or hexanoic anhydride (250 μl) to give an approximately 2-fold molar excess of anhydride over peptide. All samples were made up to 1·25 ml with H_2O and, after standing at 20°C for 18 h, solid ammonium acetate (100 mg) was added to each to destroy residual anhydride. The pH was then adjusted to 5·0 and 5 mg of pepsin added to each sample for direct application to plastein synthesis. Succinylation and esterification with methanolic HCl were achieved by established procedures (Klotz, 1967; Wilcox, 1967).

FPLC analyses were performed in a manner similar to that described earlier (Andrews *et al.*, 1985). To achieve complete solubilisation of plastein pellets, urea was added to 6M in all buffers, but mercaptoethanol, as used previously, was not necessary and was omitted. For runs at pH 7·0 or 7·5 a buffer of 50 mM Tris-HCl was employed and 50 mM sodium formate was used at pH 3·8, and 0–0·5M NaCl gradients used for elution from the ion-exchange columns.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed (Andrews, 1986) in the LKB vertical slab gel apparatus using T = 12·5%, C = 4·0% gels and the Laemmli buffer system but containing 0·2% SDS in place of 0·1% in all buffers. Peptide samples used were obtained by preparative gel filtration of pepsin digests of casein as described previously (Sukan & Andrews, 1982*a*) and corresponded closely to fractions 2, 3, 4 and 5 of Table 4 in reference (Sukan & Andrews, 1982*a*), so had average molecular weights of approximately 3000, 1300, 590 and 270, respectively. Portions of initial peptide solution and of

plastein material made from them were diluted in stacking gel buffer to 10–12 mg/ml, made 1% with respect to SDS and heated at 100°C for 2 min. Sucrose and bromophenol blue tracking dye were added and 20 μl portions applied to the sample wells in the gel slab. Control samples of pepsin only (0·1 mg/ml) were similarly treated and 20 μl applied to the gel. Amino acid analyses were performed on an LKB Model 4102 automatic analyser after hydrolysis of samples at a concentration of 1·0 mg/ml in 6N HCl at 110°C for 24 h under N_2.

RESULTS AND DISCUSSION

Requirement for proteinase catalyst

It is conventional practice for enzymic hydrolysis to be used to prepare the peptides required for the plastein reaction, but if the resulting digest is lyophilised and then made up directly into a concentrated (e.g. 20–50% w/v) solution in a buffer at a pH between about 4 and 7, the residual proteinase present is often sufficient to catalyse plastein formation with no further enzyme addition being necessary (unpublished observations). The presence of residual enzyme activity greatly complicates the interpretation of experiments aimed at elucidating the role of added proteinase in the plastein formation step. In previous work (Sukan & Andrews, 1982*a*,*b*) we attempted to overcome this difficulty by fractionating peptide hydrolysates by gel filtration or subjecting them to ultrafiltration but it is difficult to be certain that absolutely all enzyme is removed by such treatments. Therefore an experiment on plastein synthesis using peptides obtained by acid hydrolysis was performed.

Table 1 shows the clear requirement for proteinase as catalyst and it is probable that an active enzyme is needed. Pepstatin is a strong but reversible inhibitor of pepsin when operating as a hydrolase at a strongly acid pH, but its effectiveness at pH 5 is not known. Likewise, the base inactivation of pepsin at pH 12 and the heat-inactivation may also not have been completely adequate (although no remaining activity was detected by digestion of azocasein) so there was the possibility of a very low level of residual enzyme activity which might explain the small amount of plastein formed in these cases. Amounts of plastein formed were little more than 10% of that produced by pepsin itself, however. With papain, oxidation with persulphate or alkylation with iodoacetate inactivated the enzyme, but also denatured the enzyme and caused it to precipitate. This precipitate would have contributed to the pellet weight, so the amounts of true plastein formed were small, if any. Complete inactivation of the hydrolase activity of

chymotrypsin by phenylmethylsulphonyl fluoride (PMSF) was demonstrated in a preliminary experiment using hide powder azure as substrate. With this inactivated chymotrypsin, non-proteinase enzymes or other non-enzyme proteins, there was no plastein formation.

In a separate experiment 0·4 g portions of chymotryptic casein peptides dissolved in 1·0 ml of 0·2M sodium acetate buffer (pH 4·8) were incubated with 4 mg of chymotrypsin or of PMSF-treated chymotrypsin for 24 h at 37°C. While obvious turbidity developed in the former within a few minutes, and after 24 h it formed a very viscous thixotropic fluid, the latter gave only a very slight turbidity in 24 h, confirming the requirement for a catalyst of active proteinase with enzymically-produced peptides also.

Chemical modification of peptides

Peptides modified by treatment with acid anhydrides were incubated at 37°C for 65 h, by which time the controls had formed substantial amounts of plastein and were thixotropic, while the hexanoic anhydride-treated sample contained visibly less plastein, the butyric anhydride sample less still and the acetic anhydride-treated sample very little. In a separate experiment, succinylation of peptides blocked most (89·5%) of the NH_2 groups and effectively prevented plastein formation. Table 2 shows the results of a more quantitative assessment which generally agreed with these empirical observations except for the finding that hexanoic anhydride treatment led to an enhanced yield of plastein, in spite of the fact that more than one third of the NH_2 groups were blocked. This may be because hydrophobic peptides are preferentially incorporated into plastein and are the most insoluble in the phosphate washing buffer, even if not visibly precipitated as increased amounts of plastein.

The above modifications will involve substitution of both peptide α-NH_2 and ε-NH_2 groups but modification of intact casein molecules before pepsin

TABLE 2
The Effect of Chemical Modification of Peptides with Acid Anhydrides on Plastein Formation

Reagent used	*Plastein yield (mg)*	*NH_2 content (%)*[a]
None (control)	29·0	100
Acetic anhydride	5·7	23·2
Butyric anhydride	21·8	36·3
Hexanoic anhydride	79·5	63·4

[a] Control taken as 100%.

TABLE 3
Effect of Casein Modification before Hydrolysis on Plastein Yield

Modification	*Plastein yield (mg)*	*NH_2 content (%)*[a]
None (control)	131·9	100·0
Acetylation	115·8	77·3
Esterification	108·5	133·0
Acetylation + esterification	72·6	81·2

[a] Control taken as 100%.

hydrolysis will be limited very largely to side-chain ε-NH_2 groupings. The effects of acetylation, esterification and acetylation plus esterification of casein on subsequent plastein yield are shown in Table 3. Individually, acetylation and esterification caused similar small reductions in plastein yield, but in combination their effect was more than additive. There may have been a small amount of acid hydrolysis under the conditions of esterification in addition to the subsequent pepsin hydrolysis because the NH_2 group content of this material was higher than that of the control samples.

The general conclusion to be drawn from these experiments is that the changes in plastein yield do not parallel changes in either side-chain NH_2 or COOH group content or in total NH_2 group content and so are not consistent either with condensation to form new peptide bonds or with transpeptidation as reaction mechanisms for plastein formation.

Influence of organic solvents

Earlier work (Sukan & Andrews, 1982*a*,*b*) suggested that hydrophobic interactions play a major role in plastein formation, so it was of interest to examine the effects on the reaction of organic solvent addition as this should increase the solubility of hydrophobic peptides and also of the hydrophobic plastein products.

Table 4 shows the results on plastein formation (chymotrypsin catalyst) of adding various proportions of the water-miscible solvents ethanol, dimethylformamide (DMF) and glycerol to the reaction mixture containing chymotryptic peptides. Plastein was formed in all the samples and they gradually increased in viscosity during incubation, giving ultimately thixotropic gels or viscous solutions of varying consistency. All the ethanol-containing samples became noticeably more viscous than the controls in spite of the fact that slightly less plastein was formed (Table 4). The DMF-

TABLE 4
The Influence of Organic Solvent Additions on Plastein Yield

Solvent	*Plastein pellet weight (mg)*
None (control)	18·5
Ethanol: 40% (v/v)	17·0
60%	15·7
80%	12·4
Dimethylformamide: 40% (v/v)	13·2
60%	11·6
80%	7·5
Glycerol: 40% (v/v)	36·0
60%	33·9
80%	21·5

containing solutions were optically less opaque and slightly less viscous than the ethanolic samples but, as expected, the glycerol-containing ones were much the most viscous at all stages of the incubation. Ethanol addition was found (Table 4) to lead to reduced plastein formation and DMF was even more effective. However, when compared to zero solvent controls addition of glycerol led to increased amounts of plastein. The increase was greatest at the 40% level of addition.

The interpretation of these results is not clear. Increasing the proportion of any of these solvents increases the viscosity of the medium which would be expected to reduce molecular mobility and slow down the aggregation of peptides into plastein. This would explain most of the observed results but would run counter to the results with glycerol-containing solutions. These were more viscous than the other samples, so contrary to the observed results yields should have been the lowest if viscosity was the major factor involved.

In control experiments in which chymotrypsin was added to solutions of azocasein in similar solvent mixtures and the formation of azopeptides measured, it was found that glycerol had only a small effect on chymotrypsin activity but that DMF and ethanol caused greater inactivation, which was essentially total at the 80% level. Thus the influence of solvent on enzyme activity does not explain fully either the observed reductions in plastein yield caused by DMF and ethanol or the enhanced yields found in glycerol mixtures. It seems probable that the variations in yield were determined very largely by the influence of the solvent, either on the solubilities of peptides in the initial hydrolysate mixture or on the solubility of the plastein products.

Table 5 shows the influence of organic solvent addition on the amino acid

TABLE 5
Amino Acid Content of Plastein formed in the Presence of Organic Solvents (n moles/100 μg)

	Hydrophobic	*Hydrophilic*	*Other*
Initial casein	418·0	373·3	93·3
Control	458·6	308·2	100·9
40% DMF	308·7	454·7	155·8
60% DMF	302·2	447·8	165·0
80% DMF	286·6	452·2	185·3
40% Ethanol	308·9	449·3	142·3
60% Ethanol	305·5	474·0	142·9
80% Ethanol	293·0	443·4	159·8
40% Glycerol	406·1	378·2	100·7
60% Glycerol	402·6	381·9	96·5
80% Glycerol	387·3	389·6	106·2

composition of the plasteins formed. It can be seen that, compared with the initial casein hydrolysate, the control plastein formed in aqueous buffers was enriched in hydrophobic amino acids (Phe, Leu, Ile, Tyr, Val, Pro) and depleted in the more hydrophilic amino acids (Asp, Glu, Ser, Thr, Lys, Arg), in agreement with previous results (Sukan & Andrews, 1982*a*). Surprisingly, in the presence of organic solvents, this situation was reversed and the plasteins contained a lower proportion of hydrophobic amino acids than in the initial digest and very much lower than in the control plastein, while conversely the hydrophilic amino acid content was increased. These differences were least and quite small when the organic solvent was glycerol but both ethanol and DMF had a dramatic effect. In all cases nearly all the change seen was found at the 40% level of solvent addition and increasing it further (up to 80%) caused little further change. While grouping the amino acids in this way was convenient, there were some differences in behaviour of individual amino acids within each group (data not shown). For example, Ile and Val showed smaller reductions than the other hydrophobic amino acids in the plasteins from solvent mixtures, while Arg increased less than the other hydrophilic residues and the weakly hydrophobic residues Gly and Ala (grouped under Other in Table 5) increased substantially.

Plastein stability

To measure stability, samples of plastein were prepared from peptic peptides in the usual way and harvested by centrifugation (3000*g*, 20 min). Two peptide fractions obtained by preparative gel filtration on Sephadex G-25, having average peptide molecular weights of about 1000 and 3000, were used

as starting material. After centrifugation the supernatant was discarded and the pellets freed of unincorporated peptide by two washes involving resuspending the pellets in 5 volumes of 0·2M sodium acetate buffer (pH 4·8) and recentrifugation. The final pellet was then resuspended in one volume of buffer and reincubated for 48 h. After centrifugation, absorbance readings of the supernatant at 230 nm showed that 25·5% and 19·5% of the plastein pellets from the high and low molecular weight peptide fractions, respectively, had dissociated from the pelleted material. Removal of supernatant, two washings of the pellets as above and then a further reincubation of the resuspended residual plastein pellets, but this time also in the presence of 2·5 mg/ml of pepsin, showed that a further 20·5% and 14·5%, respectively, of the two samples were solubilised by such treatment. These experiments showed clearly that plastein formation was reversible and that this did not appear to be influenced significantly by the presence of proteinase.

In a repeat experiment the washed plastein pellets were reincubated at 37°C for 24 h in 0·1M sodium acetate buffer (pH 4·8) in the presence of small amounts (2 mg/ml) of dabsyl-labelled peptide. Approximately 90% of the dabsyl peptides became associated with the preformed plastein, even when no proteinase catalyst was present. This high level of incorporation (compared with 18% and 22% incorporation of unlabelled peptides into plastein in the initial stage) was consistent with the increase in peptide hydrophobicity on addition of the dabsyl groups and the fact that hydrophobic peptides are preferentially incorporated into plastein. Experiments similar to the above, however, showed that, in spite of this strong association, the process was reversible and 10·0% and 7·6% of the dabsyl peptides (as shown by absorbance readings at 480 nm) dissociated from the plasteins formed from high and low molecular weight peptides, respectively, during a reincubation of the washed plastein. The occurrence of this dissociation under such mild conditions showed that peptides had been incorporated by physical bonds and not via covalent bond formation.

These observations and conclusions were confirmed when the initial peptide mixtures contained a small proportion (4%) of coloured dabsyl-labelled peptide so that they were included into plastein from the beginning. It was found that 84·7% and 85·8% were incorporated into the plasteins formed from high and low molecular weight peptide fractions, respectively. In accordance with this preferential involvement of hydrophobic peptides only small amounts (generally 1·5–2·5%) were dissociated by reincubating the washed plastein pellets as above. Even the additions of substantial amounts (50 mg/ml) of fresh unlabelled peptide to the incubation mixtures were ineffective competitors for binding and did not significantly influence the dissociation of dabsyl peptides.

FPLC experiments

Samples of pepsin-, papain- and chymotrypsin-derived peptides from total casein, before plastein formation, and of the plastein pellet made from them (using the same enzyme for the hydrolysis and plastein synthesis stages in all cases) were examined by ion-exchange chromatography using the FPLC apparatus. When the cation-exchange Mono Q column was used at pH 7·5, very little of the peptides adhered to the column. Rather more peptide adhered to the column when the anion-exchange Mono S column was used under the same conditions, but on reducing the pH to 3·8 there was a further marked improvement. In all cases the patterns of eluted peaks, in initial peptide mixtures and in the redissolved plastein pellet fraction, were qualitatively similar, although there were quantitative differences (Fig. 1). However, in every sample only a minor portion of the peptide adhered to the column, sometimes only 15–20%, and most passed straight through

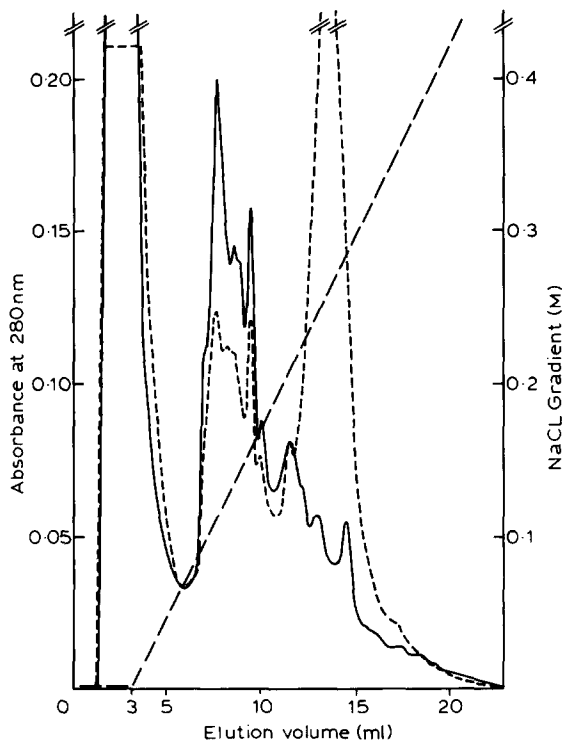

Fig. 1. FPLC separation on a Mono S column at pH 3·8 of peptides obtained from total casein by 48-h hydrolysis with papain (——) and of the redissolved plastein pellet material obtained from them (– – –) using papain as the synthesis catalyst. (See text for experimental conditions.)

unhindered, so the significance of these results was uncertain as the behaviour of this portion may not be truly representative of the whole sample. Nevertheless, no new peptide peaks appeared to be formed on plastein production, which was most consistent with a physical aggregation mechanism.

Gel filtration runs, at pH 7·0 on the Superose 12 column, were more satisfactory in that nearly all sample material was dissolved in the urea-containing buffer before application to the column. Again, there were no qualitative differences and only very slight quantitative differences between the peptide peak profiles of the initial peptide mixtures or of the redissolved plastein pellets (Fig. 2). This was true whether pepsin, chymotrypsin or papain was used (same enzyme employed for both hydrolysis and synthesis stages).

These three proteinases were also used in experiments where each of the three enzymes was used for each stage (i.e. nine experiments in total). Visual inspection of the sample mixtures after 18 hours' incubation at 37°C revealed that plastein had formed in all nine samples, but that most was

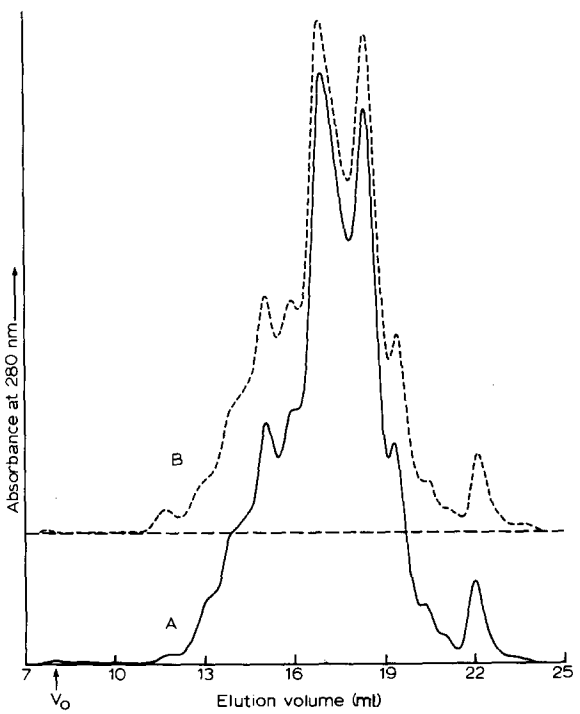

Fig. 2. Gel filtration analysis by FPLC with a Superose 12 column of (A) the peptides in a chymotrypsin digest of total casein (——) and (B) of the redissolved plastein pellet material produced from them (– – –) using chymotrypsin as the synthesis catalyst. (See text for details.)

TABLE 6
Influence of Choice of Proteinase for Hydrolysis and Synthesis on Plastein Yield

Proteinase		*Plastein yield (mg dry wt/g peptide)*	*Visual observation after 72 h incubation (37°C)*
Hydrolysis	*Synthesis*		
Chymotrypsin	Papain	39·5	Slightly transparent thixotropic viscous liquid
Chymotrypsin	Pepsin	126·7	Thixotropic gel
Chymotrypsin	Chymotrypsin	32·0	Thixotropic gel, easily broken to viscous liquid
Pepsin	Papain	20·0	Opaque liquid
Pepsin	Pepsin	73·8	Opaque thixotropic viscous liquid
Pepsin	Chymotrypsin	21·4	Opaque liquid
Papain	Papain	56·5	Opaque thixotropic thick viscous liquid
Papain	Pepsin	68·6	Opaque thixotropic viscous liquid
Papain	Chymotrypsin	40·6	Slightly transparent liquid

formed in those with pepsin as polymerisation catalyst. Chymotryptic peptides appeared to be the best substrate (chymotryptic peptides plus pepsin was the only sample to have actually formed a thixotropic gel at this stage), followed by papain peptides with peptic hydrolysates being the least favourable. After 72 h at 37°C the incubation was terminated and plastein yield measured (Table 6). Qualitative visual inspection agreed with the quantitative pellet weights and pepsin was found to be the best catalyst of plastein synthesis. In contradiction to the visual results, however, peptides produced by papain appeared to be a better substrate for the synthesis reaction than chymotryptic peptides, unless pepsin was used as the synthesis catalyst, in which case the chymotryptic peptides were best. The reasons for this are not clear but may reflect the different specificities of the enzymes as hydrolases. If the same enzyme is used in both hydrolysis and synthesis stages there will be little further hydrolysis during the synthesis step but, if dissimilar enzymes with different bond specificities are used, it is an obvious possibility. The superior synthetic abilities of pepsin may result from its lack of hydrolytic activity at pH 4·8. FPLC gel filtration of the supernatant fractions after plastein formation supported this (Fig. 3). When chymotrypsin was used to catalyse plastein synthesis from peptic peptides there was a clear shift in peptide size to lower molecular weights which was not seen when pepsin itself was employed. This shift was even more marked when papain was used. Figure 3 also confirms that when the pepsin-catalysed plastein pellet was redissolved the peptide profile was qualitatively identical to that of the unincorporated supernatant peptides, although there may be some preferential incorporation of the larger peptides. Very similar results

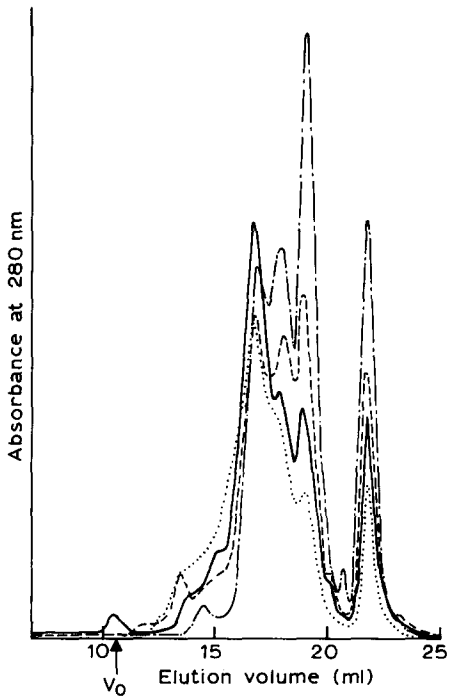

Fig. 3. FPLC gel filtration (Superose 12 column) analysis of the supernatant fractions obtained when the peptides obtained by pepsin digestion of total casein were used as substrate for plastein synthesis, using the same or different proteinases as catalyst for the synthetic reaction. Pepsin (——), chymotrypsin (– – –) or papain (– · – · –). The profile obtained with the redissolved plastein pellet, using pepsin as catalyst for both stages, is shown for comparison (· · · ·) and is clearly similar to that of the pepsin plastein supernatant fraction.

were found for chymotryptic peptides (Fig. 4), pepsin causing some further hydrolysis and papain causing even more. This 'extra hydrolysis' caused by using dissimilar enzymes in the two stages was greatest when pepsin peptides were used as the substrate for plastein synthesis, less with chymotryptic peptides and least with papain hydrolysates. There was no evidence for the formation, during plastein synthesis, of covalently-bonded high molecular weight material that resisted dissolution in urea-containing buffers. This was confirmed by FPLC gel filtration runs in buffers containing 0·1% added sodium dodecyl sulphate (SDS) in which all the plasteins were entirely soluble.

SDS-PAGE

SDS-PAGE analysis of peptic peptide fractions and plastein pellets derived from them is shown in Fig. 5. Most of the peptides in all fractions were quite

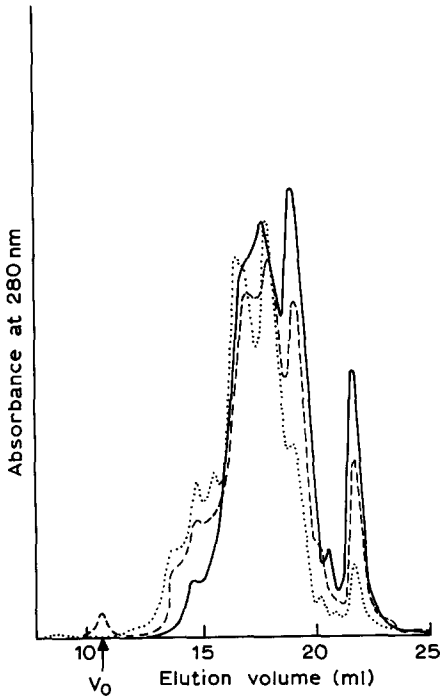

Fig. 4. FPLC gel filtration (Superose 12 column) analysis of the supernatant fractions obtained when chymotryptic peptides of casein were used as substrate for plastein synthesis, using chymotrypsin (····), pepsin (– – –) or papain (——) as catalyst for the synthetic reaction.

small and migrated together as a zone close to the buffer front but a few migrated as discrete slower bands. While not ideal, therefore, it was considered that SDS-PAGE would be very suitable for detecting the formation of any covalently-linked high molecular weight components. From Fig. 5 it was obvious that no such material was formed during plastein synthesis, and no new peptide or protein zones were detected in any fraction, the only high molecular weight zones seen being due to the added pepsin. Indeed, far from synthesising new peptide zones or even heterogeneous streaks of peptides with increased molecular weight, there was some further hydrolysis of the peptide zones that were originally present.

CONCLUSIONS

All our findings are consistent with the association of peptides into an insoluble plastein product being an entirely physical process. In spite of the fact that substantial amounts of insoluble material precipitate out of the reaction mixture, the process is essentially reversible. There was no evidence

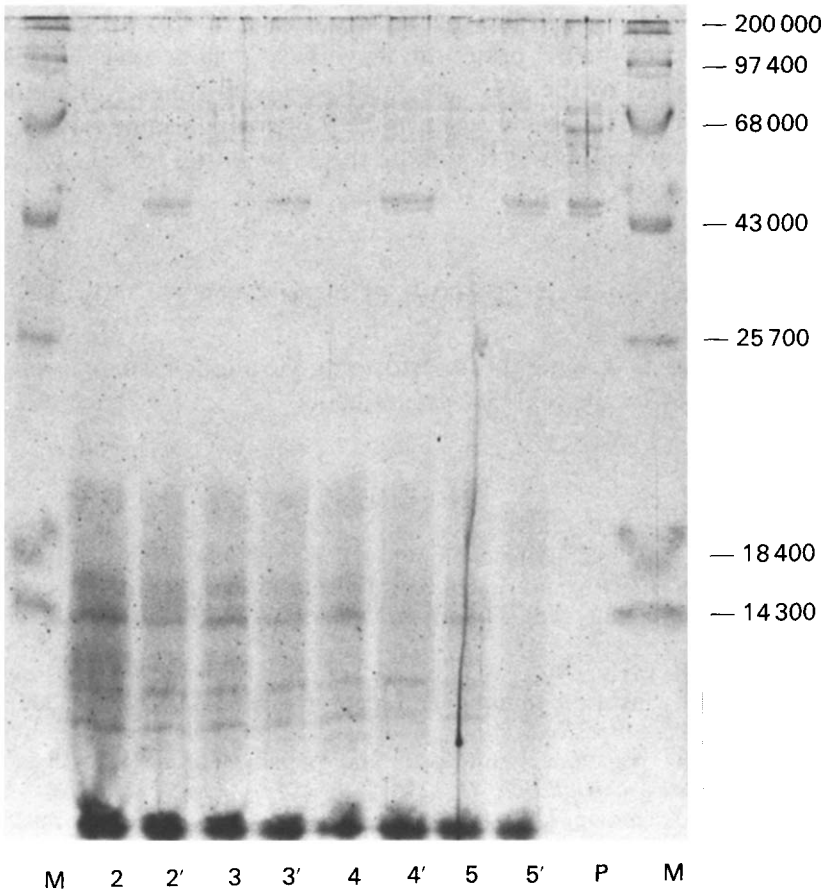

Fig. 5. SDS-PAGE analysis of fractions (2, 3, 4, 5) of peptic peptides prepared by gel filtration (Sephadex G-25 column) from total casein, and of plastein pellet material (2′, 3′, 4′, 5′) prepared from them using pepsin as the synthesis catalyst (see text for details). P, control sample of pepsin only. M, molecular weight marker proteins (Bethesda Research Laboratories Ltd).

for any covalent bond formation, either via peptide bond synthesis or a transpeptidation mechanism. Preliminary experiments in which plastein synthesis was followed by small-angle neutron scattering (SANS) showed scattering at very small angles but little change at larger angles. This suggested that there were unlikely to be long-range interactions or the formation of a network structure but rather a growth of small particles into larger ones, as occurs during a simple aggregation process. The requirement for a proteinase to catalyse the synthesis has been clearly shown and active enzyme is probably obligatory, although it is possible that inactivated or inhibited proteinase may also be capable of acting as a rather inefficient

catalyst. The role performed by the enzyme is far from clear, however, particularly since the pH optimum of synthesis may be very different from that of hydrolysis by the same enzyme. It seems most likely that binding of peptide at or close to the active site of the proteinase in some way can form a template or nucleation site to initiate the aggregation process, but further work is required to establish this.

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