Protease-Catalyzed Formation of Plastein Products and Some of Their Properties

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Enzymatic hydrolysates were prepared from whey protein concentrate and fish protein concentrate using a new alkali-stable protease, Esperase. Solutions containing high concentrations of ultrafiltered peptides were incubated with Esperase or Alcalase under conditions leading to the formation of water-insoluble, so-called plastein products. These could be dissolved in 8 M urea, 6 M guanidine, 50% acetic acid, or 2 M perchlorate. Sephadex chromatography of hydrolysates and plastein products dissolved in strong acetic acid showed that only slight changes in the molecular weight distribution of the peptide mixtures occurred during plastein formation. No material of protein character with high molecular weight was formed. It is concluded that plastein reactions leading to gel formation may be described as a kind of rearrangement process in which transpeptidation and hydrolysis play a more important role than condensation reactions. The driving force in the overall process is the formation of precipitates and gel matrices in which relatively small peptides are associated by means of noncovalent bonds.

Proteases (peptidyl-peptide hydrolases, EC 3.4.4) normally catalyze the hydrolysis of internal bonds in proteins and peptides giving rise to products which have lower molecular weights than the substrates. These fragments are usually soluble in water. Proteases are therefore used in the preparation of protein hydrolysates intended for incorporation in beverages and liquid foods (Reed, 1975). If, however, highly concentrated solutions of protein hydrolysates are incubated with proteases, water-insoluble and gel-forming products may be formed. These products, usually called plasteins, were discovered almost 100 years ago, but the mechanism of their formation has been very controversial.

Fujimaki et al. (1971) have suggested that plastein reactions may have several applications in food manufacturing, and they have studied conditions for plastein formation in great detail. By following the increase in material insoluble in trichloroacetic acid (Cl_3CCOOH) or ethanol these authors have found that plastein reactions usually occur most rapidly between pH 5 and 7 even with proteases having a more acidic or alkaline pH optimum for the normal hydrolytic reaction. The rate of the reactions has been found to be influenced by the concentration and average molecular weight of the peptide solution used as substrate.

The formation of plastein has usually been considered as a reversal of normal hydrolytic reactions and to be caused by the exceptionally high substrate concentration (usually at least 30%) during the reaction. As support for this hypothesis Determann and Eggenschwiller (1965) showed that direct condensation of some simple synthetic peptides to oligomers may be catalyzed by pepsin at high substrate concentrations. Data have also been presented which would indicate that plastein products have very high molecular weights. Tauber (1951) obtained plastein products by incubating peptic digests of various proteins with chymotrypsin and assumed from ultracentrifugation studies that protein-like substances with a molecular weight of 250000–500000 were formed. A more recent study by Yamashita et al. (1974) suggested that the upper limit of the molecular weight of a peptic hydrolysate of soybean globulin increased from 2600 to 21600 during incubation with chymotrypsin. However, the reported values on the molecular weights of plastein products cannot be considered reliable due to their poor solubility in water and ordinary buffers.

From their studies of plastein reactions catalyzed by chymotrypsin, Horowitz and Haurowitz (1959) concluded that direct condensation of peptides played only a minor role and that transpeptidation was the dominating type of reaction. Fujimaki and co-workers have discussed the relative importance of transpeptidation and condensation reactions and they have recently suggested that hydrophobic interaction may be an important factor in the production of the water-insoluble products (Arai et al., 1975). However, they still define plastein formation as a polycondensation process giving rise to products of high molecular weight.

We have carried out plastein reactions by incubating partial enzymatic hydrolysates of whey and fish protein concentrates with bacterial proteases of low specificity and characterized the products by chromatography on Sephadex. Our results show that peptide chain elongation plays only a minor role in the formation of gels and water-insoluble products, and that noncovalent bonds between peptides of relatively low molecular weight are responsible for the peculiar properties of the reaction products.

EXPERIMENTAL SECTION

Substrates and Enzymes. Whey protein concentrate (WPC) containing 70–75% protein and approximately 12% lactose prepared from ordinary sweet rennet whey by gel filtration (Lindquist and Williams, 1973) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Fish protein concentrate (FPC) containing about 80% protein was obtained from Astra Nutrition, Mölndal, Sweden.

Proteases were obtained from Novo Industri A/S, Copenhagen, Denmark. Esperase is a new bacterial protease of wide specificity and with exceptionally high stability and activity in the alkaline pH region. A preparation having a specific activity of 9.5 Anson units/g was used for making protein hydrolysates, whereas a crystalline product having an activity of 30 Anson units/g was used in the plastein experiments. Alcalase is a subtilisin-type protease prepared by submerged fermentation of a *Bacillus* sp. The preparation used had a specific activity of 6 Anson units/g.

Preparation of Protein Hydrolysates. The hydrolysis of WPC and FPC with Esperase was first studied in small

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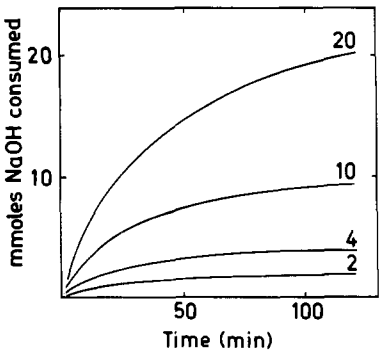

Figure 1. Hydrolysis of whey protein concentrate (WPC) with Esperase at 50 °C. Alkali consumption at pH 10.0 was followed by means of a pH stat. The numbers denote the amounts of WPC in grams per 200 ml of water. The enzyme:substrate ratio was 1:400.

scale experiments carried out in a pH stat (Radiometer, Copenhagen, Denmark). The hydrolysis was carried out at pH 10.0 in a nitrogen atmosphere, and the consumption of 0.1 M NaOH was recorded after addition of the enzyme. Figure 1 shows the hydrolysis of different amounts of WPC at a constant enzyme:substrate ratio (1:400). The total alkali consumption was approximately proportional to the substrate concentration when the reaction had ceased. Addition of more enzyme did not cause the reaction to resume.

Larger batches of hydrolysates were prepared in a similar manner by incubating 100 g of WPC or FPC in 2000 ml of water with 250 mg of Esperase. The reactions were stopped by circulating ice-cold water through the flask and adjusting the pH to 7.0 with HCl. It was possible, in this manner, to obtain more or less hydrolyzed preparations of reproducible composition. The ice-cold neutralized solutions were centrifuged in the cold to remove small amounts of insoluble material. They were then passed through an Amicon Hollow Fibre Cartridge Type PM10 run at 4 °C. This ultrafilter is specified as retaining molecules having a molecular weight over 10000. However, small amounts of Esperase corresponding to 1–2% of the enzyme concentration were found to leak through the filter. The ultrafiltrates were freeze-dried.

Plastein Reactions. The freeze-dried hydrolysates were dissolved in water and the pH adjusted, usually to 6.0. For chromatography experiments, 0.5-ml aliquots were mixed in small tubes with a few microliters of a protease solution or water and incubated in a water bath, usually at 50 °C. The reactions were stopped by addition of 0.5 ml of acetic acid which dissolved the more or less turbid gels which had been formed.

Yamashita et al. (1970) used the rate of formation of Cl_3CCOOH-insoluble material as an assay for studying plastein reactions. We used a similar procedure incubating 0.25 ml of the hydrolysate solutions with enzyme and adding 20 ml of 10% Cl_3CCOOH in water after which the turbidity was read at 660 nm. A very slight turbidity was obtained before enzyme was added, and it then increased at an initially constant rate.

Sephadex Chromatography. A glass column (bed dimension: 135 × 1.5 cm) was packed with Sephadex G-50 Fine (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 50% (v/v) analytical grade acetic acid in water. Samples (0.5–1.0 ml) of hydrolysates and plastein reaction products containing the same amount of material in 50% (v/v) acetic acid were applied. Elution was carried out at 20 °C with the same solvent by means of a peristaltic pump. Fractions of constant volume (usually 5 ml) were collected, and the material distribution in the eluates was determined either by measuring the absorbance at 280 nm or by carrying out ninhydrin reactions (Moore, 1968) on aliquots before or after hydrolysis in 6 M HCl as described earlier (v. Hofsten, 1956). The column was calibrated by means of a number of reference substances of known molecular weight dissolved in 50% (v/v) acetic acid.

A series of plastein experiments was done with a partial hydrolysate of WPC which had been group-fractionated on a large Sephadex G-50 column (bed dimension: 70 × 7 cm). Samples (10 g) of freeze-dried hydrolysate dissolved in 5% acetic acid were applied, and elution was done with the same solvent. The eluate was pooled into three fractions, I, II, and III, which roughly corresponded to the three peaks A, B, and C in the analytical experiment illustrated in Figure 2b. These solutions were freeze-dried.

RESULTS AND DISCUSSION

General Conditions for Plastein Formation from Protein Hydrolysates. The ultrafiltered and freeze-dried protein hydrolysates could be dissolved in water at concentrations up to 50% (w/w). When solutions containing at least 30% (w/w) were adjusted to pH 6 and incubated with Esperase or Alcalase, water-insoluble and gel-forming products were formed within a few minutes or about an hour depending on the enzyme concentration and incubation temperature. Slow gel formation occurred at 50 °C within 10–24 h also in the absence of added enzyme, because the hydrolysates were contaminated with small amounts of Esperase, which had leaked through the ultrafilter.

It was difficult to study the rate of plastein reactions by simply observing the solutions because these gradually became more viscous. At substrate concentrations above 30% (w/w) such stable gels were formed that when test tubes were inverted, the contents did not flow out. However, the gels were thixotropic and became fluid when the tubes were vibrated or shaken vigorously.

Yamashita et al. (1970) used a turbidimetric assay based on the formation of Cl_3CCOOH-insoluble material for studying plastein reactions. We tried a similar procedure and found that the reaction had a pH optimum around 6 for both Esperase and Alacalase, and that the rate was dependent on the amount of enzyme added. With Esperase the initial reaction rate increased at pH 6 up to temperatures of about 80 °C.

Solubility of Plastein Products. Gels and precipitates formed during plastein reactions were almost insoluble in water or buffer solutions of varying ionic strength in the pH range 3–9. We found, however, that they could be completely dissolved at room temperature in acetic acid of a final concentration of about 50% (v/v). It was also possible to dissolve the plastein products in 8 M urea or 6 M guanidine at neutral pH. The exact mechanism by which these solvents affect the solubility of peptides and proteins is not known, but it is likely that they interfere with both hydrogen bonds and hydrophobic forces which hold the molecules together into water-insoluble complexes.

Highly chaotropic ions such as 2 M sodium perchlorate completely solubilized plastein products from WPC hydrolysates at pH 6–7. This is a further indication that noncovalent bonds are involved in the formation of plastein gels and precipitates. The well-known solubilizing effect of chaotropic ions on membrane proteins is not yet fully understood, but it is considered that such ions facilitate the transfer of apolar residues to the aqueous phase and lead to the destabilization of multimeric protein complexes (Hatefi and Hanstein, 1974).

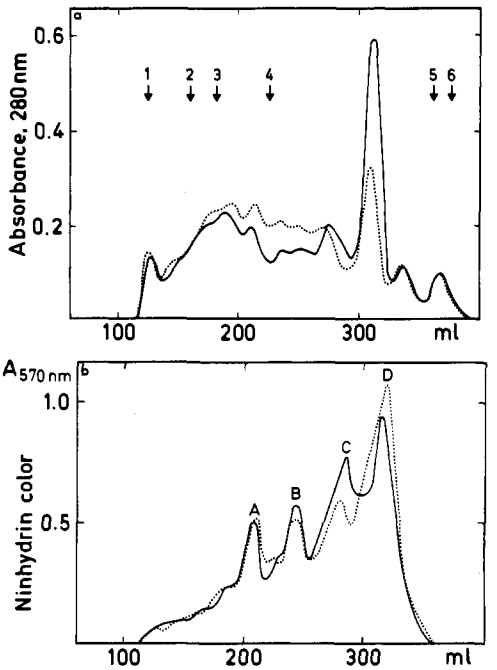

Figure 2. Chromatography on Sephadex G-50 of 90 mg of a partial hydrolysate of WPC before and after plastein formation. The column (135 × 1.5 cm) was equilibrated and eluted with 50% (v/v) acetic acid. The material distribution in the eluates was estimated by measuring the absorbance at 280 nm (a) and by the direct ninhydrin method (b): (solid lines) WPC hydrolysate; (dotted lines) plastein product obtained after incubation for 2 h at 50 °C of a 45% (w/w) solution of the hydrolysate with Esperase at an E/S ratio of 1/400. The numbers denote elution positions of the following compounds: (1) chymotrypsinogen (mol wt 25 000) and lysozyme (mol wt 14 400); (2) insulin (mol wt 5733); (3) B-chain of insulin (mol wt 3300); (4) bacitracin (mol wt 1422); (5) tyrosine; (6) NaCl.

Plastein gels could be dissolved by adjusting their pH to above 10 with small amounts of alkali. Gels were again formed if the pH was adjusted to below 7. The plastein products could also be freeze-dried without losing their ability to gel when dissolved in the original volume of water.

Molecular Weight Distribution of Peptides in Hydrolysates and Plastein Products from WPC. Our finding that gels and precipitates formed in plastein reactions were soluble in acetic acid made it possible to estimate the molecular weight distribution of the substrates and products by chromatography on Sephadex G-50. Preliminary experiments showed that much better resolution of peptide mixtures was obtained with 50% (v/v) acetic acid than with ordinary buffers. One reason for this is that the adsorption of aromatic peptides to Sephadex is reduced with this solvent (Eaker and Porath, 1967). Peptide bonds are not hydrolyzed at room temperature in acetic acid, and we found no changes in the elution profiles of protein hydrolysates and plastein products after storage in 50% (v/v) acetic acid for several days.

A series of experiments was done with a freeze-dried WPC hydrolysate which had been obtained after incubation with Esperase until about 30% of the maximal alkali consumption had been reached. Figure 2 shows elution profiles on Sephadex G-50 of this material before and after a plastein reaction carried out for 2 h at 50 °C and a substrate concentration of 45% (w/w). The material distribution was determined both by reading the absorbance at 280 nm (Figure 2a) and by carrying out ninhydrin reactions on aliquots of the fractions collected (Figure 2b). The Sephadex column had previously been calibrated by means of some peptides and proteins of known molecular weight, and it is evident that no protein-like material of high molecular weight was formed during the plastein reaction. The uv-absorbance curves show that a high peak containing small aromatic peptides was reduced leading to a corresponding increase in peptides of slightly higher molecular weight. The elution profiles obtained after ninhydrin analysis were similar before and after the plastein reaction.

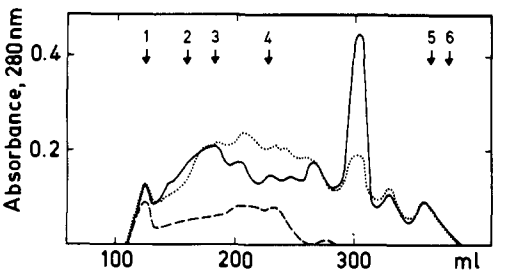

Figure 3. Sephadex chromatography of 60 mg of WPC hydrolysate before and after a plastein reaction at 30% (w/w) substrate concentration: (dashed line) Cl_3CCOOH insoluble material after plastein reaction. Other symbols and experimental conditions are as in Figure 2.

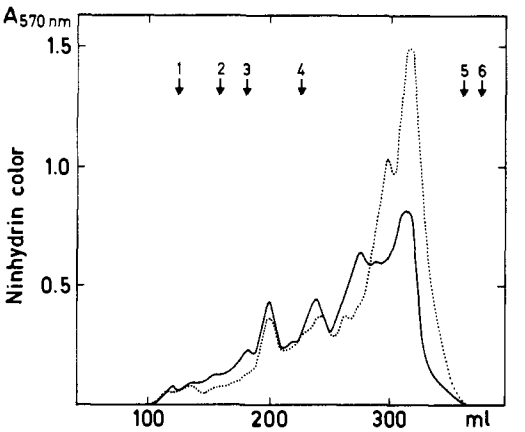

Figure 4. Sephadex chromatography of 40 mg of WPC hydrolysate before and after plastein formation at 20% (w/w) substrate concentration. Experimental conditions and symbols are as in Figure 2.

Gel was formed after only about 5 min under the described conditions for the plastein reaction. When the reaction was terminated by addition of acetic acid after different times (up to 24 h) no significant changes in the elution profiles were obtained.

The same WPC hydrolysate was used for plastein reactions carried out at lower substrate concentrations. Figure 3 shows that the peak containing small aromatic peptides also diminished when the substrate concentration was 30% (w/w). The substrate was soluble in 10% Cl_3CCOOH, whereas a precipitate was formed when the reaction product was mixed with Cl_3CCOOH. This precipitate was washed with Cl_3CCOOH, dissolved in a small volume of 50% (v/v) acetic acid, and chromatographed on the Sephadex column. The elution profile of this material shows that the Cl_3CCOOH-insoluble material contained peptides of a molecular weight down to 1000–2000.

Figure 4 shows that a plastein reaction carried out at 20% (w/w) substrate concentration gave rise to an increase in the amount of small peptides reacting with ninhydrin. A gel was also formed after about 4 h under these conditions, but it was soft. At 10% (w/w) substrate con-

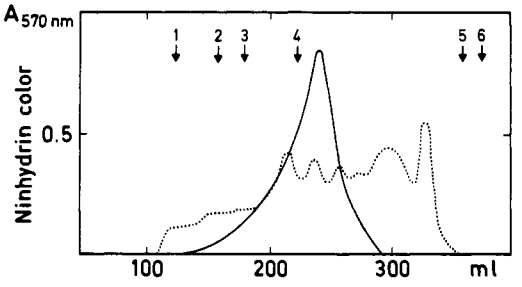

Figure 5. Sephadex chromatography of a group-fractionated WPC hydrolysate containing peptides of relatively high molecular weight: (solid line) distribution of ninhydrin reacting peptides before plastein reaction; (dotted line) plastein product obtained after incubation with Esperase at 40% (w/w) substrate concentration.

centration, hydrolysis was predominant, leading to a marked increase in the amounts of small ninhydrin-reacting peptides. No gel but a slight precipitate was formed after several hours.

The experiments described were repeated with Alcalase. There was no change in the appearance of the plastein products, and the elution profiles on Sephadex were similar to those obtained after plastein reactions with Esperase.

Plastein Formation from Fractionated WPC Hydrolysate. Our own results as well as those of Fujimaki et al. (1971) indicated that the average molecular weight of the peptides in protein hydrolysates influenced the properties of the plastein products. We thus found that a hydrolysate, which had been prepared by exhaustive digestion of WPC with Esperase, gave slower reactions and softer gels than partial hydrolysates. In order to study this effect more closely, a sample of a WPC hydrolysate similar to that illustrated in Figure 2 was group-fractionated on a large column of Sephadex. The eluate was pooled into three fractions (I, II, and III) of different average molecular weight, and these were freeze-dried and used for plastein reactions carried out at a substrate concentration of 40% (w/w).

Figure 5 shows that fraction I, containing peptides of relatively high molecular weight, yielded a plastein product additionally containing larger and smaller peptides. Relatively slight changes in the elution pattern were observed when fraction II containing intermediate size peptides had been incubated with Esperase. Fraction III, which contained small peptides, gave a plastein gel, in which peptides of increased molecular weight were found. Figure 6A shows that these newly formed peptides contained aromatic amino acids because a new uv-absorbing peak was obtained. Direct ninhydrin analysis of the eluate (Figure 6B) showed that the new peptides gave a low color yield. When aliquots of each fraction were acid hydrolyzed before the ninhydrin reaction, a more complex elution profile was obtained (Figure 6C). This kind of analysis ought to give a more true picture of the quantitative distribution of peptides in the eluate, and the curve indicates that the newly formed peptides have a molecular weight of 2000–3000.

Formation of Gels at High pH. The turbidimetric assay showed that Cl_3CCOOH-insoluble material was formed most rapidly during plastein reactions carried out around pH 6. However, gels were formed also when concentrated solutions of WPC hydrolysate were incubated with Esperase or Alcalase in the alkaline pH region, where these enzymes are optimally active in normal hydrolytic reactions. One experiment was done in which a 45% (w/w) solution of a partial WPC hydrolysate was adjusted to pH 10.4 before addition of Esperase. The solution had formed

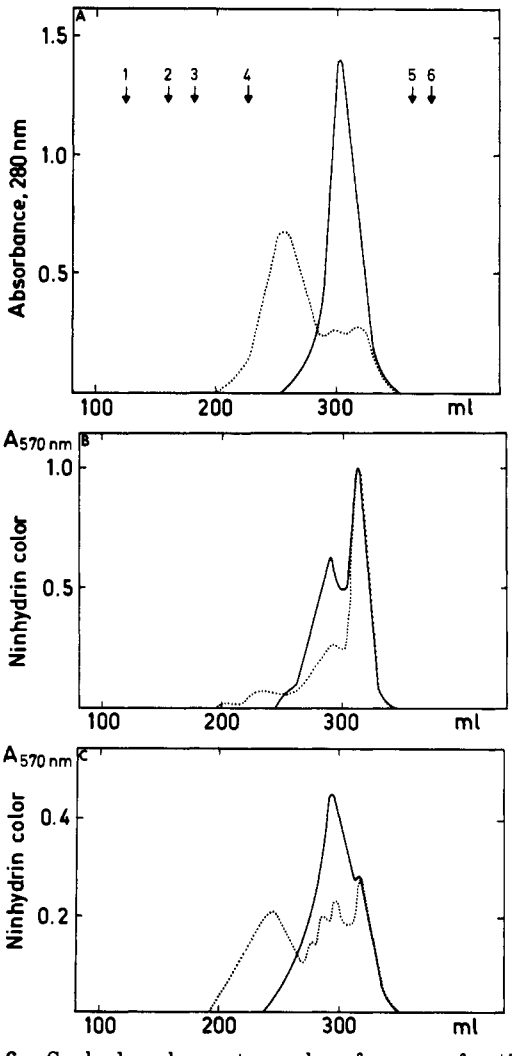

Figure 6. Sephadex chromatography of a group-fractionated WPC hydrolysate containing peptides of relatively low molecular weight: (solid lines) distribution of peptides before plastein reaction; (dotted lines) plastein product obtained after incubation with Esperase at 40% (w/w) substrate concentration; (A) material distribution estimated by reading the absorbance at 280 nm; (B) direct ninhydrin analysis of the eluates; (C) ninhydrin analysis of eluate after hydrolysis in 6 M HCl.

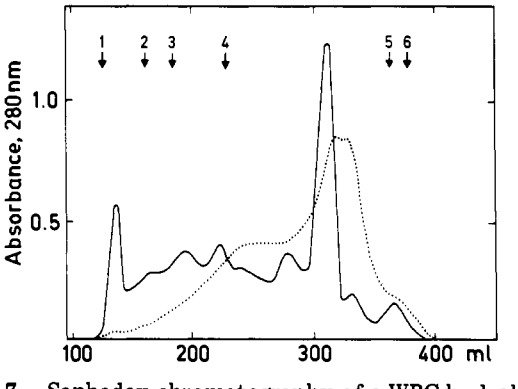

Figure 7. Sephadex chromatography of a WPC hydrolysate before and after incubation with Esperase at an alkaline pH. The substrate concentration was 45% (w/w) and the initial pH was 10.4 falling to 8.4 after 2 h incubation at 50 °C: (solid line) WPC hydrolysate; (dotted line) product after incubation with Esperase.

a gel after about 2 h incubation at 50 °C; the pH had then decreased to 8.4 showing that proteolysis had occurred. The gel was dissolved in an equal volume of acetic acid and

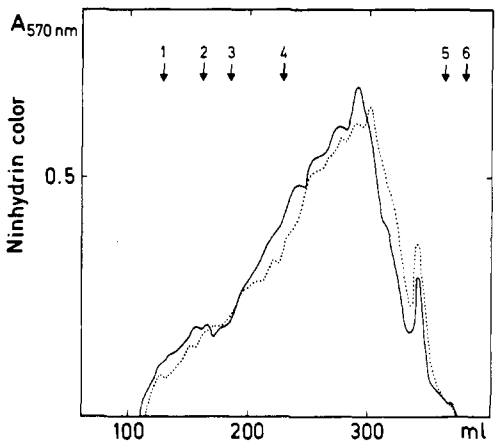

Figure 8. Sephadex chromatography of a partial enzymatic hydrolysate of fish protein concentrate before and after plastein reaction: (solid line) distribution of ninhydrin reacting peptides in hydrolysate; (dotted line) gel-forming plastein product obtained after incubation with Esperase at 40% (w/w) substrate concentration.

chromatographed on Sephadex G-50. Figure 7 shows the elution pattern of the peptide solution before and after this reaction, and it illustrates that a marked proteolysis has occurred. No increase in the amount of high molecular weight material was obtained, and the formation of gels from peptide solutions is thus no indication that a condensation process has taken place.

Plastein Experiments with Fish Protein Concentrate. Fujimaki et al. (1971) have earlier described the preparation of plastein products from enzymatic hydrolysates of codfish. Onoue and Riddle (1973) have also suggested that fish waste can be converted to a high-quality protein-like product by the digestion with pepsin and subsequent plastein reactions with the same enzyme.

We prepared hydrolysates of a commercial fish protein concentrate (FPC) in the same manner as the WPC hydrolysates. Water-insoluble and gel-forming products were obtained when concentrated solutions of such hydrolysates were incubated with Esperase or Alcalase. Figure 8 illustrates elution profiles on Sephadex G-50 of a partial hydrolysate before and after a plastein reaction carried out at 50 °C for 2 h at a substrate concentration of 40% (w/w). Only slight changes in the molecular weight distribution of the ninhydrin-reacting peptides occurred during the reaction. No material of high molecular weight was formed.

CONCLUSIONS

A plastein reaction has usually been described as a reversal of normal proteolysis. Our experiments show that the gels and precipitates formed during incubation of concentrated peptide solutions with a protease of wide specificity are composed of relatively small molecules held together by noncovalent bonds. The expression "plastein reaction" may be useful in describing the overall process resulting in such water-insoluble products. The term plastein ought, however, to be used with similar caution as the trivial name peptone because it does not denote any well-defined type of substance. Both plasteins and peptones are complex mixtures of peptides, the difference being mainly in their solubility properties.

At least three different types of reactions may be catalyzed by proteases when added to peptide solutions. Condensation reactions, which may be considered as reversed hydrolytic reactions, would lead to a marked decrease in the number of ninhydrin reacting groups, and this was not observed in our experiments. Such reactions are also likely to play only a minor role in plastein formation because they are thermodynamically and sterically unfavorable. At high pH values, hydrolysis is the dominating type of reaction catalyzed by alkaline proteases even if the substrate concentration is high. It appears that there is a shift toward transpeptidation when the incubation is carried out in the neutral or slightly acid pH region. It is not known if the specificity of a protease is influenced when the reaction is carried out at an abnormal pH, but our finding that aromatic peptides are preferentially recombined to slightly larger molecules may indicate this.

The reactions taking place during plastein formation may perhaps be described as a kind of rearrangement process where the equilibrium is influenced by the formation of peptides tending to form insoluble complexes. Hydrophobic interaction between peptide side chains apparently plays an important role in the formation of such complexes, but electrostatic interaction may also be involved. Aso et al. (1974) have found that there is an increase in the proportion of hydrophobic amino acids in the water-insoluble reaction product in comparison with the substrate. The proportion between hydrophilic and hydrophobic peptides in the incubation mixture clearly influences the properties of plasteins (Arai et al., 1975).

The physical chemistry of protein gels is very complex (Tombs, 1974). The gel network is apparently held together by hydrogen bonds, and it is probable that the makeup of plastein gels is similar.

Further studies on the mechanism of plastein reactions and the accompanying formation of gels may give information of value for food technology. It is particularly interesting that plasteins have a less bitter taste than soluble protein hydrolysates (Fujimaki et al., 1971). It is also possible to enhance the essential amino acid content in plasteins by carrying out the reactions in the presence of the corresponding amino acid esters (Yamashita et al., 1972).

The presence of active enzymes in plastein products may cause problems in food applications. We used the bacterial protease Esperase in our experiments because it is advantageous to carry out the hydrolysis at a high pH (Archer et al., 1973) and this enzyme also gave very rapid plastein formation. Further studies are needed to evaluate its use in the production of hydrolysates and plasteins.

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LITERATURE CITED

Arai, S., Yamashita, M., Aso, K., Fujimaki, M., *J. Food Sci.* **40**, 342 (1975).
Archer, M. C., Ragnarsson, J. O., Tannenbaun, S. R., Wang, D. I. C., *Biotechnol. Bioeng.* **15**, 181 (1973).
Aso, K., Yamashita, M., Arai, S., Fujimaki, M., *J. Biochem. (Tokyo)* **76**, 341 (1974).
Determann, H., Eggenschwiller, M. W., *Justus Liebigs Ann. Chem.* **690**, 182 (1965).
Eaker, D., Porath, J., *Sep. Sci.* **2**, 507 (1967).
Fujimaki, M., Kato, H., Arai, S., Yamashita, M., *J. Appl. Bacteriol.* **34**, 119 (1971).
Hatefi, Y., Hanstein, W. G., *Methods Enzymol.* **31**, 770 (1974).
Hofsten, B. v., *Acta Chem. Scand.* **10**, 1432 (1956).
Horowitz, J., Haurowitz, F. J., *Biochim. Biophys. Acta* **33**, 231 (1959).
Lindquist, L. O., Williams, K. W., *Dairy Ind.* **38**, 459 (1973).
Moore, S., *J. Biol. Chem.* **243**, 6281 (1968).
Onoue, V., Riddle, V. M., *J. Fish. Res. Board Can.* **30**, 1745 (1973).
Reed, G., Ed., "Enzymes in Food Processing", 2nd ed, Academic Press, New York, N.Y., 1975.

Tauber, H., *J. Am. Chem. Soc.* **73**, 1288 (1951).
Tombs, P. M., *Discuss. Faraday Soc.* **57**, 158 (1974).
Yamashita, M., Arai, S., Aso, K., Fujimaki, M., *Agric. Biol. Chem.* **36**, 1353 (1972).
Yamashita, M., Arai, S., Aso, K., Fujimaki, M., *Agric. Biol. Chem.* **38**, 1269 (1974).
Yamashita, M., Arai, S., Matsuyama, J., Gonda, M., Kato, M., Fujimaki, M., *Agric. Biol. Chem.* **34**, 1484 (1970).

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Biological Inactivation of Proteins by the Maillard Reaction. Effect of Mild Heat on the Tertiary Structure of Insulin

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An average of 3.7 hexose residues bound the reactive amino groups of insulin during 120 days of storage with D-[U-^{14}C]glucose at 37 °C and 70% relative humidity. Dimethylaminonaphthalenesulfonylation of insulin reacted for 15 days corroborated the preferential binding at the N-terminal residues (A_1-Gly and B_1-Phe). While the acid solubility increased tenfold at 15 days, suggesting dissociation of the hexamer into dimers, there was little or no change in its structural conformation as attested to by two biological functions. The 15-day Maillard insulin retained 78.2% of the ability to depress the level of blood glucose in rabbits, and 100% of its capacity to raise blood tryptophan in young rats. These results contrast with those for the reaction at 55 °C in which a large number of sugar residues have been demonstrated to bind the protein molecule during 1 month of storage.

In the Maillard reaction, reducing sugars form condensation products with the amino groups of amino acids, peptides, and proteins. At the initial stages, α-*N*-1-deoxy-2-ketohexose derivatives have been isolated and characterized from the reaction of glucose with amino acids (Heyns and Noack, 1964) and peptides (Heyns and Rolle, 1959). The Maillard reaction of proteins has been studied mostly on food proteins which appear to lose considerable solubility, digestibility, and biological value during the course of the reaction. In previous studies we found that egg albumin reacted under mild conditions (37 °C, 68–70% relative humidity) lost 50% of its nutritive value in the first 10 days, long before the appearance of any detectable discoloration (Tanaka et al., in press). We have suspected, therefore, that irreversible loss of nutritive value could occur in food proteins prior to the observation of extensive physicochemical changes.

Insulin is a well-defined small protein in which the early steps of the Maillard reaction could be studied. Schwartz and Lea (1952) determined that in the insulin–glucose system, the order of reactivity at 37 °C is B_1-phenylalanine > A_1-glycine > B_{29}-lysine but no mention was made of the stoichiometry of the reaction. In an effort to elucidate the structure of the brown pigment, Clark and Tannenbaum (1974) used insulin to demonstrate that up to 31 hexose residues could bind and cross-link the reactive amino groups of the polypeptide after browning for 37 days at 55 °C.

A study of the first physicochemical events which may bear on the biological properties of a Maillard protein must be made under reaction conditions that result in minimal polymerization and cross-linking at the reactive amino groups. The objective of this work was to modify crystalline insulin by the Maillard reaction under mild conditions (37 °C), measure the average number of hexose residues bound to the protein in the span of 4 months, and estimate the extent of early structural changes by observing the performance of the hormone in two different biochemical functions.

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EXPERIMENTAL SECTION

Browning of Insulin. Reaction mixtures were prepared in 20-ml glass vials by suspending 50 mg of crystalline insulin (bovine pancreas, 0.5% zinc, Sigma Chemical Co., St. Louis, Mo.) in 10 ml of 7.75% glucose, and lyophilizing to a final temperature lower than 10 °C. The vials were then stored uncapped in sealed chambers at 37 °C and 68% relative humidity for up to 4 months. For the glucose binding studies, each vial also contained from 20 to 50 μCi of D-[U-^{14}C]glucose (International Chemical and Nuclear Co., Cleveland, Ohio).

Glucose Binding. For every time point, the contents of a vial were dissolved in 10 ml of an acetic acid solution (approximately 5%, pH 2.6), and 0.3 ml was loaded onto a Bio-Gel P-6 (200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) column, and eluted with the same acetic acid solution. Other conditions for the gel filtration were: column size, 1 × 50 cm; flow rate, 22 ml/h; void volume, 18 ml; fraction volume, 1.5 ml. Fifty microliters of each fraction was counted in a liquid scintillation counter. The ninhydrin pattern was obtained after hydrolysis of 0.15 ml of each fraction with 1 ml of 2.5 N KOH at 121 °C for 40 min, followed by neutralization with 30% acetic acid. Unreacted glucose was determined in the fractions by the Glucostat method (Worthington Bio-Chemicals, Freehold, N.J.).

Glucose-free Maillard insulin samples (10–15 nmol) were dansylated (8-dimethylamino-1-naphthalenesulfonylated) and analyzed by the method of Gros and Labouesse (1969) using Sequanal grade urea (Pierce Chemicals, Rockford, Ill.). For this procedure the thin-layer chromatography (TLC) plates were spotted, developed, and scraped promptly to minimize the loss of fluorescence. The fluorescent material collected from the spots was quan-