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# Plasteins. Their Preparation, Properties, and Use in Nutrition

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The theoretical and practical aspects of the plastein reaction, which consists in the formation of a gel following the addition of an endopeptidase to a concentrated solution of a partial protein hydrolysate, are examined and the properties and possibilities of using plasteins in nutrition are discussed in detail. It is shown that valuable protein food products can be obtained with the aid of the plastein reaction from proteins with an unbalanced aminoacid composition and from chemically synthesised amino-acids. Other applications of plasteins in nutrition are discussed and the studies carried out hitherto on the mechanism and driving forces of plastein formation are considered.
The bibliography includes 143 references.

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## I. INTRODUCTION

One of the most important taks of the food industry at the present time is to provide the rapidly growing world population with balanced food products, in the first place protein food products. The proteins used by man as food must be non-toxic, must be free from toxic or other undesirable impurities, and must have a balanced amino-acid composition, i.e. must contain in the first place the essential aminoacids† in the required amounts and proportions[1,2]. Animal proteins fully meet the above requirements, but the sharp increase in their production necessary to satisfy man's needs encounters many economic and technical difficulties. The task of wide-scale employment of proteins derived from non-traditional sources, for example, from sources of plant or microbiological origin, is becoming increasingly urgent.

However, such proteins usually contain toxic and other undesirable impurities and have an unbalanced aminoacid composition. For a wide-scale employment in man's food of proteins from non-traditional sources, it is necessary, in the first place, to free these proteins from impurities and, in the second place, to balance their aminoacid composition. In order to improve the aminoacid composition, it is as a rule necessary to increase their content of essential aminoacids. From the economic point of view, the use of synthetic aminoacids, which can be obtained in large amounts from cheap raw materials, is of greatest promise[3]. Since the presence of free aminoacids in protein food products has a negative influence on their food value, it is necessary for the added essential aminoacids to be present in the final product in the form of polypeptides consisting of one or different aminoacids. One of the ways of obtaining pure protein products with a high food value from contaminated proteins with an unbalanced aminoacid composition and from chemically synthesised aminoacids involves the use of the so-called plastein reaction.

† The aminoacids which are not synthesised in the human organism and must be supplied as food products are referred to as essential. They include isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Histidine is an essential amino-acid only for children[2].

Almost 100 years ago Denilevskii found that, when gastric juice is added to a concentrated solution of the enzymatic hydrolysate of any protein, an opaque solution is formed after a time and is gradually converted into a gel[4]. Later Zav'yalov suggested that the reaction be referred to as the plastein reaction and that the gel be called plastein[5]. Up to the end of the 1950's, the plastein reaction was regarded as a possible way of synthesising proteins in the human organism, but, after the discovery of the true mechanism of protein biosynthesis[6-9], studies on plastein formation temporarily ceased. Interest in the plastein reaction grew again at the beginning of the 1970's, because in many instances it proved to be convenient for the preparation of food products.

The plastein reaction is most likely to be used for the following practical purposes: (1) purification of proteins and the formation of protein products with the necessary aminoacid composition; (2) the elimination of the bitter taste from enzymatic protein hydrolysates; (3) the preparation of protein pastes and gels with a high food value for use as fillers in various food products and capable of being used independently as food; (4) the preparation of mixtures of peptides with a definite smell, taste, and other characteristics for use as food additives and odorants.

Various aspects of the plastein reaction have been examined in a number of reviews[10-17]. They show that, although the ways for the practical employment of plasteins have already been defined on the whole, much remains obscure, particularly from the theoretical standpoint. The present review has been written to summarise the studies carried out in this field hitherto and to examine the further prospects of the plastein reaction.

## II. CONDITIONS AND REAGENTS NECESSARY FOR THE PLASTEIN REACTION

The plastein reaction takes place in solutions of peptides with a molecular weight between 500 and 1500[5,22-30] at a concentration exceeding 10%[18-21] and a pH of the reaction mixture between 4 and 7.[22,23,31-33]

It can probably be now regarded as established that plasteins are obtained when any endopeptidase acts on hydrolysates of virtually any proteins. The hydrolysates can be obtained both by treatment with any endopeptidase and by partial non-enzymatic hydrolysis of protein. The influence of the properties of the enzyme and protein employed on plastein formation will be examined below in the Section devoted to the influence of various factors on the plastein reaction. One should note that the problem of which properties of a particular plastein can be regarded as common to all plasteins and which depend on the method of synthesis of the plastein has not been studied experimentally hitherto.

## III. PHYSICAL PROPERTIES AND STRUCTURE OF PLASTEINS

### 1. The Structure of Plasteins

The structure of plasteins resembles that of denatured proteins. X-Ray diffraction studies on plasteins revealed the presence of linear polypeptides with close-packed chains extended to the maximum extent[23,34]; structures resembling the secondary and tertiary structures of the protein from the hydrolysates of which they have been obtained have not been detected in the plasteins investigated. The fact that the structure of the initial protein is not restored on plastein formation is confirmed by the absence of enzymatic activity in plasteins obtained from pepsin and trypsin hydrolysates[35] and also by the absence of hormonal activity in plasteins obtained from insulin[36]. The immunological activity of certain plasteins can be explained by the entrainment of a certain amount of the active enzyme and of impurities from the initial protein[11,37-40]. Denaturing agents do not affect the solubility of plasteins, which shows that they do not have an ordered structure[41]. A tertiary structure of the type of an $\alpha$-helix can probably exist only in plasteins enriched in glutamic acid where the content of the latter is 42% against 24% in the initial protein. The circular dichroism spectra of solutions of such plasteins indicate the possibility of the formation of oligomers with an $\alpha$-helix structure from glutamic acid residues[42]; the circular dichroism spectra of the usual plasteins do not indicate the presence of such structures. It has been suggested at one time that cyclic polypeptides play a major role in the structure of plasteins[43,44]. Such hypotheses have not been put forward in studies devoted to the plastein reaction in recent years.

Thus the data enumerated above suggest that plasteins consist of a conglomerate of intertwined polypeptide chains with different lengths and structures.

### 2. The Properties of Plasteins

Despite the fact that plasteins differ from the initial proteins in their secondary and tertiary structures, they resemble proteins in many chemical and physical properties. They are cleaved by various proteolytic enzymes such as endopeptidases and exopeptidases[20,25,42,45-47]. Plasteins have the lowest solubility in the region of the isoelectric point and the dependence of their solubility on the pH of the solution is similar to that for the initial protein[48]. The isoelectric point has been determined for plasteins obtained by treating with $\alpha$-chymotrypsin the pepsin hydrolysate of soya-bean protein. It has been shown by the method of isoelectric focussing that it is located in the pH range between 3.7 and 4.8.[42,48] Isoelectric focussing of plastein enriched in glutamic acid showed that it has several isoelectric zones in the pH range from 1.5 to 4.0. Plasteins obtained from human serum albumin and casein proved to be electrophoretically homogeneous, in contrats to the initial proteins, and their electrophoretic mobility is somewhat lower than that of the initial proteins. Plasteins synthesised from various proteins differ somewhat in their electrophoretic mobilities[43,49-51]. Like proteins, plasteins react with ninhydrin to form coloured compounds, form complexes with $Cu^{2+}$ ions, and are precipitated by typical protein precipitants such as trichloroacetic acid or various dyes[20,44,52-55].

### 3. The Molecular Weights of Plasteins

The molecular weights of plasteins have been frequently determined by various methods, but the problem of the molecular weight which plasteins can acquire and the factors on which it depends has not been finally solved. The molecular weights and the methods used for their determination are listed in Table 1.

**Table 1.** Molecular weights of plasteins and methods of their determination.

| Molecular weight | Method | Refs. |
|---|---|---|
| Hydrolysate 685-1045, plastein 25000 | Polyacrylamide gel electrophoresis (PAGE) | 22 |
| Plastein < 21000 | PAGE | 32 |
| Plastein > 1200 | PAGE; precipitation with trichloroacetic acid and dyes | 41 |
| Hydrolysate 1280, plastein without additives 12050, plastein enriched in glutamic acid 6240 | Amino-nitrogen determined by Van Slyke method | 42 |
| Plastein < 1000 | Ultracentrifugation | 44 |
| Plastein 250000-500000 | Ultracentrifugation | 57 |
| Plastein ~5000 | Ultracentrifugation | 58 |
| Hydrolysate 1550, plastein 5490 | Amino-nitrogen determined by the Van Slyke method | 59 |
| Plastein without additives 12000, plastein enriched in glutamic acid 6500 | PAGE | 60 |
| Plastein enriched in methionine 12000 | Amino-nitrogen determined by the Van Slyke method | 61 |
| Molecular weight remains unchanged on plastein formation | Chromatography on Sephadex | 62 |
| Hydrolysate 1089, plastein without additives 2748, plastein enriched in tryptophan 3019, plastein enriched in threonine 2832, plastein enriched in lysine 2791 | Amino-nitrogen determined by Van Slyke method | 63 |
| Plastein 5000-6000 | Amino-nitrogen determined by the copper complex and cryoscopic methods | 64<br>65 |
| Plastein ~1000 | Ultracentrifugation | 66 |
| Plastein 3000 | Cryoscopy | 67 |
| It has been shown that plasteins do not contain low-molecular-weight peptides in the absence of substrate | Chromatography on Sephadex | 68 |

One should note that the extremely high molecular weights found by Tauber[57] are probably due to the pronounced ability of plasteins to aggregate; as a result of this, one determines the molecular weight of aggregates linked via hydrophobic interactions and not of the polymeric molecules themselves, from which the plasteins are made up[48]. As regards other molecular weights obtained it is difficult to say what factors are responsible for the observed differences. The only regularity is that plasteins enriched in glumatic acid have the lowest molecular weights. Plasteins are polydisperse[58] and it is most

likely that in the majority of cases their molecular weights are in the range from 5000 to 15 000.[41,42,58-61] However, there exist fairly reliable data showing that the molecular weight does not change on plastein formation[42,52,62]. It appears that, under various process conditions, either the polycondensation reaction, which increases the molecular weight, or transpeptidation, in which the molecular weight remains unchanged, predominates.

It is not possible to state exactly at the present time which particular conditions are responsible for the alteration of the plastein formation mechanism, which leads to the formation of plastein specimens with different molecular weights. The factors suggested in various studies as responsible for the change in the mechanism of the plastein reaction will be examined further in the text below.

## 4. The Aminoacid Compositions and Solubilities of Various Plastein Fractions‡

Plasteins are on the whole sparingly soluble in all the solvents and mixtures investigated. Unfortunately in the majority of the studies the solubility of plasteins was studied only qualitatively. It can be assumed that at approximately pH 4.0 the solubility of plasteins in water is less than 1%.[42] The higher the concentration of the hydrolysate from which the plastein is formed, the greater the weights of the water-soluble fraction[47].

**Table 2.** Dependence of the solubility of plastein synthesised with pepsin from the pepsin hydrolysate of soya-bean protein on the pH of the solution[48].

| pH | $A^*_{600}$ | pH | $A_{600}$ | pH | $A^*_{600}$ | pH | $A_{600}$ |
|---|---|---|---|---|---|---|---|
| 2.0 | 0.155 | 4.5 | 0.307 | 6.0 | 0.253 | 10.0 | 0.009 |
| 4.0 | 0.294 | 5.0 | 0.267 | 8.0 | 0.206 | — | — |

* Turbidimetric determination; $A_{600}$ is the optical density of the plastein suspension at 600 nm.

The solubility of plasteins has been studied as a function of the pH of the solution, its ionic strength, and the concentrations of urea, guanidinium chloride, and sodium dodecyl sulphate[47,48,53,62]. The dependence of the solubility of plasteins synthesised with pepsin from the pepsin hydrolysate of soya-bean protein on pH is illustrated in Table 2. Evidently the plastein investigated has a solubility minimum at pH ~4, i.e. in the region of the isoelectric point[42]. Presumably all plasteins therefore have a solubility minimum at pH 3-4, because the isoelectric points of the majority of peptides are located in this precise region. Data for the solubilities of plasteins in concentrated acids and alkalies are contradictory. It has been shown[20,53] that certain plasteins dissolve in concentrated nitric and hydrochloric acids. On the other hand, there are data indicating the opposite behaviour[24]. Plasteins apparently dissolve more effectively in concentrated alkalies[20,24,50].

It has been noted that the solubility of plasteins depends little on the ionic strength in neutral solutions, increasing only by 40% with increase of ionic strength from 0.1 to 1.[48] However, it is most likely that the dependences of the solubilities of different plasteins on ionic strength are different. The salting out of plasteins from their solutions in 0.025 M aqueous sodium hydroxide following the addition of NaCl has been noted[53]. In certain cases it has been observed that the dependence of the solubility of plasteins on ionic strength is represented by a curve with a minimum corresponding to an ionic strength of 0.5.[78]

**Table 3.** Aminoacid composition of various plastein fractions obtained with the aid of pepsin from the pepsin hydrolysate of soya-bean protein[41].

| Aminoacid | Aminoacid content in given fraction, wt.%* | | | |
|---|---|---|---|---|
| | № 1 | № 2 | № 3 | № 4 |
| Asp | 17.92 | 11.21 | 5.42 | 11.83 |
| Glu | 37.22 | 7.21 | 5.89 | 14.90 |
| Lys | 5.73 | 6 03 | 6.65 | 3.54 |
| Arg | 3.25 | 3 20 | 1.12 | 1.76 |
| His | 1.10 | 1.50 | 0.62 | 0.87 |
| Met | 0.74 | 1.70 | 0.95 | 0.38 |
| Cys | 0.73 | 2.26 | 1.21 | 1.34 |
| Trp | 0.63 | 2 47 | 0.79 | 1.72 |
| Gly | 5.79 | 5.26 | 5.10 | 3.52 |
| Ser | 6.93 | 5.95 | 4.75 | 7.24 |
| Thr | 2.05 | 3.72 | 2.55 | 2.53 |
| Ala | 2.67 | 4.95 | 6.02 | 3.34 |
| Val | 2.00 | 5.39 | 8.41 | 4.60 |
| Leu | 4.51 | 16.91 | 31.00 | 22.34 |
| Pro | 4.84 | 10.83 | 7.70 | 5.39 |
| Tyr | 0.63 | 1.23 | 1.32 | 1.84 |
| Ile | 1.76 | 4.40 | 7.61 | 5.96 |

* Fraction No. 1 dissolves in neutral buffer solutions; fraction No. 2 does not dissolve in neutral buffer solutions but dissolves in sodium dodecyl sulphate solution; fraction No. 3 does not dissolve in either neutral buffer solutions or in sodium dodecyl sulphate solution; fraction No. 4 dissolves in 50% aqueous ethanol but does not dissolve in water.

The solubility of plasteins increases markedly in aqueous solutions of urea, guanidinium chloride, and sodium dodecyl sulphate, the effect of a 6 M aqueous solution of urea or guanidinium chloride being equilvalent to that of a 0.002 M aqueous solution of sodium dodecyl sulphate[37,48]. Certain plasteins[62] readily dissolve also in 50% aqueous acetic acid and in 2 M aqueous sodium perchlorate at pH 6-7. The exact mechanism of the action of these solvents on plasteins and proteins is unknown, but it is extremely likely that they decrease the hydrophobic interactions, which promotes the transfer of non-polar aminoacid residues to the aqueous phase[79,81].

‡ The aminoacid compositions of the initial proteins and various plastein fractions, isolated mainly on the basis of their solubilities in water, have been published[19,41,42,48,52,57,63,69-77]. One should note that the term "aminoacid composition of plasteins" is usually understood as the aminoacid composition of the fraction of the plastein formation product isolated in a particular way. The aminoacid composition of plasteins as a whole should not change. When the aminoacid compositions of various plastein fractions are compared, the influence of the method of fractionation on the aminoacid composition of the fraction is as a rule disregarded, which can lead to incorrect conclusions concerning the nature and mechanism of the formation of plasteins.

The increase of the solubility of plasteins under the influence of substances which reduce hydrophobic interactions suggests that plasteins are sparingly soluble in water precisely owing to the hydrophobic properties of their polypeptide chains. Consequently an increase of the content of hydrophobic aminoacids in the water-insoluble fractions should be observed. The data in Tables 3 and 4 show that there is more of hydrophobic aminoacids in the fraction insoluble in sodium dodecyl sulphate solution than in the fraction insoluble in the buffer solution. The content of hydrophobic aminoacids also increases in the fraction soluble in 50% aqueous ethanol but insoluble in water. In other words, the lower the solubility of the plastein fraction in water, the higher its content of hydrophobic aminoacids regardless of the enzyme and the particular hydrolysate used to obtain the plastein. All these findings suggest that a low solubility of plasteins is due to the accumulation of hydrophobic aminoacids in the sparingly soluble fraction[48].

Table 4. Aminoacid composition of the plastein fraction* soluble in 50% aqueous ethanol but insoluble in water (p), relative to the aminoacid composition of the initial hydrolysate (S)[74].

| Amino-acid | S | P | P/S |
|---|---|---|---|
| Asp | 1.25 | 0.85 | 0.69 |
| Glu | 1.35 | 0.84 | 0.62 |
| Lys | 0.87 | 0.68 | 0.78 |
| Arg | 0.40 | 0.39 | 0.98 |
| Gly | 1.00 | 1.00 | 1.00 |
| Ser | 0.76 | 0.79 | 1.04 |
| Thr | 0.62 | 0.57 | 0.92 |
| Ala | 1.31 | 1.32 | 1.01 |
| Val | 0.76 | 1.18 | 1.55 |
| Leu | 0.91 | 2.98 | 3.27 |
| Pro | 0.23 | 0.68 | 2.44 |
| Phe | 0.45 | 1.39 | 3.09 |
| Tyr | 0.50 | 0.60 | 1.20 |
| Ile | 0.62 | 1.15 | 1.85 |

*The plasteins were synthesised with the aid of bioprase from the pepsin hydrolysate of baker's yeast protein. The weight content of glycine was assumed to be unity.

A difference in the aminoacid composition is also observed in the fractions separated from the reaction mixture by ultrafiltration or dialysis, i.e. fractions consisting of peptides having a molecular weight higher than a certain definite value. The data in Table 5 show that in this case the test fraction also exhibits an increase of the content of hydrophobic aminoacids and a decrease of that of hydrophilic aminoacids relative to the initial hydrolysate, although the effect is not so marked.

Thus the solubility of any fraction of plasteins is presumably determined primarily by its aminoacid composition, the plastein formation process involving a rearrangement of the aminoacid residues with formation of a sparingly soluble fraction having an increased content of hydrophobic aminoacids. The possibility of a change in the aminoacid composition of the high-molecular-weight fraction of plasteins on addition of ethyl esters of aminoacids to the initial reaction mixtures has been demonstrated. Typical changes in the aminoacid composition in this instance are presented in Table 6. Evidently the aminoacid composition of the high-molecular-weight fraction can change fairly considerably relative to the aminoacid composition of the same fraction of the given plastein obtained in the usual way - without the addition of the ethyl esters of aminoacids to the reaction mixture. The mechanism of this process and the possible ways of employing such plasteins will be discussed in the following Sections. For the moment, we shall merely point out that the specimens of high-molecular-weight fractions of plasteins obtained, which are enriched in glutamic acid, are much more soluble than either the plasteins with the unchanged composition or the initial protein[42].

Table 5. Aminoacid composition of the high-molecular-weight fraction of plastein obtained by treating the papain hydrolysate of zein with papain, relative to the aminoacid composition of the initial protein[63].

| Amino-acid | Aminoacid content, wt.% | | γ* | Amino-acid | Aminoacid content, wt.% | | γ* |
|---|---|---|---|---|---|---|---|
| | protein | plastein | | | protein | plastein | |
| Asp | 4.61 | 3.91 | 0.85 | Ser | 4.42 | 4.28 | 0.97 |
| Glu | 21.70 | 13.02 | 0.60 | Thr | 2.40 | 2.13 | 0.89 |
| Lys | 0.20 | 0.19 | 0.95 | Ala | 7.56 | 8.77 | 1.16 |
| Arg | 1.56 | 1.74 | 1.11 | Val | 3.62 | 6.20 | 1.71 |
| His | 1.07 | 1.06 | 0.99 | Leu | 20.18 | 23.49 | 1.16 |
| Met | 1.58 | 2.04 | 1.29 | Pro | 10.93 | 9.37 | 0.86 |
| Cys | 1.00 | 0.92 | 0.92 | Phe | 6.63 | 6.98 | 1.05 |
| Try | 0.32 | 0.33 | 1.03 | Tyr | 4.73 | 5.54 | 1.17 |
| Gly | 1.48 | 1.52 | 1.03 | Ile | 4.39 | 5.67 | 1.29 |

*$\gamma$ is the ratio of the content of aminoacids in the plastein specimen to their content in the protein.

Table 6. The increase in the content by weight ($\gamma$) of certain aminoacids in the high-molecular-weight fractions (molecular weight > 500) of plasteins derived from various proteins* when the ethyl esters of the aminoacids are introduced into the reaction mixture[19].

| Amino-acid | Number of protein | | | | |
|---|---|---|---|---|---|
| | № 1 | № 2 | № 3 | № 4 | № 5 |
| Lys | — | 1.36 | 1.69 | 1.37 | 9.29 |
| Glu | 2.80 | — | — | — | — |
| Met | 6.76 | 8.40 | 6.0 | 2.79 | — |
| Try | — | 1.81 | 1.94 | 1.25 | — |

*Glutamic acid was used in the form of the $\alpha$, $\gamma$-diethyl ester. When the plastein reaction is carried out in the usual way, the content of these aminoacids in the plastein fraction investigated decreases. Proteins from soya (protein No. 1), clover *Trifolium repens* L. (protein No. 2), seaweed *Spirulina maxima* (protein No. 3), bacterial protein from *Phodopseudomonas capsulatis* (protein No. 4), and gluten (protein No. 5) were used.

The data in Table 7 show that the introduction of glutamic acid makes it possible to increase significantly the solubility of plasteins. The solubility of plasteins enriched in glutamic acid is not influenced by heating the solution at 60°C for 30 min, while the solubility of the usual plasteins changes by a factor of three under the same conditions[42]. This indicates a major contribution of hydrophobic interactions to the formation of plasteins[82-84]. The increased solubility and thermal stability of plasteins enriched in

glutamic acid are probably due to their comparatively low molecular weight and the increased content of glutamic acid as a result of which the hydrophobic interactions in such plasteins are greatly weakened.

**Table 7.** Dependence on the pH of the solution of the solubilites at room temperature of soya-bean protein (A), plastein synthesised with the aid of papain from the pepsin hydrolysate of soya-bean protein (B), and plastein enriched in glutamic acid and obtained from the same hydrolysate with the aid of papain (C). The glutamic acid contents were 24% in (A) and (B) and 42% in (C)[42].

| Specimen | Percentage content of nitrogen in solution relative to the total nitrogen of the sample in a 1% mixture at the pH values indicated | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 9 |
| A | 75.1 | 80.2 | 70.3 | 12.5 | 18.3 | 44.4 | 83.7 | 97.5 |
| B | 90.2 | 88.5 | 81.0 | 75.0 | 77.2 | 80.3 | 83.5 | 87.4 |
| C | 98.1 | 98.0 | 97.6 | 97.5 | 97.7 | 98.0 | 98.3 | 98.3 |

## 5. The Rheological Properties of Plasteins

The rheological properties of plasteins have been studied systematically in relation to the plastein obtained with the aid of bioprase from the pepsin hydrolysate of soya-bean protein[18]. It was shown that the plastein gels obtained are thixotropic and that the rheological properties change in the direction of greater plasticity and elasticity with increase of the initial concentration of the substrate. This supports the view that plasteins are typical coagulation structures[85], i.e. structures obtained as a result of aggregation via van der Waals forces. The main condition for the formation of coagulation structures is an inhomogeneous mosaic structure–the presence of relatively hydrophobic sections of the chain on the surface of the particles coated by solvation shells. Point contacts–primary units of the structure–arise on these hydrophobic sections. Indeed studies have shown that hydrophobic forces are in fact the cause of gel formation when plasteins are synthesised[41,48].

Thus the gel formation reaction in the preparation of plasteins belongs to the third class of solution–gel transitions[86]. This class of transitions includes processes in which a gel is formed as a result of the loss of solubility by the dissolved substance on altering the temperature or the solvent or when a chemical reaction takes place. In the initial substrate solution all the species are hydrated. A chemical reaction results in the formation of species which exhibit a smaller tendency towards hydration, which makes their aggregation a thermodynamically favourable process as a result of the increase of the entropy of the system[82,83,87].

It follows from the rheological properties of the plastein specimens obtained at different initial substrate concentrations that the most cross-linked structure is observed for specimens obtained from a 35% solution of the hydrolysate[18]. On further increase of substrate concentration, the plastein reaction product solidifies and begins to exhibit the properties of condensation-crystallisation structures[85]. The great strength of the structures obtained from substrate solutions at an initial concentration in excess of 35% is confirmed by the finding that such specimens do not separate into layers with liberation of water even on centrifugation for 30 min at 6500 g at room temperature[18,88].

It is of interest that the plastein gels obtained hardly manifest the phenomenon of syneresis.

The relative viscosities of various plastein specimens have been investigated as a function of their concentration in solution or suspension[18]. The results of the study of the relative viscosities of 5% suspensions of specimens are presented in Table 8. The viscosity of the P-10 specimen is lower than that of the hydrolysate, which suggests that, at a substrate concentration of 10%, the substrate undergoes appreciable hydrolysis and this entails a decrease of viscosity. The sharp fall of the viscosity from the P-35 to the P-45 specimen is apparently a consequence of a qualitative rearrangement of the plastein structure from the coagulation type to the condensation-crystallisation type at an initial substrate concentration in excess of 35%.

**Table 8.** Relative viscosities $\eta$ of a 5% solution or suspension of soya-bean protein, the pepsin hydrolysate of soya-bean protein, and various specimens of plasteins obtained from the pepsin hydrolysate of soya-bean protein with the aid of bioprase[18]*.

| Specimen | Hydrolysate | Soya-bean protein | P-10 | P-20 | P-25 | P-30 | P-35 | P-45 |
|---|---|---|---|---|---|---|---|---|
| $\eta$ | 13 | 33 | 9.3 | 12.5 | 15 | 15.8 | 19 | 14 |

*Specimens of plasteins obtained from hydrolysates with initial substrate concentrations of 10, 20, 25, 30, 35, and 45% were investigated. The specimens were designated by P-10, P-20, P-25, P-30, P-35, and P-45 respectively.

In order to understand the driving forces of the plastein reaction, we shall consider how important is the formation of a precipitate and a gel on plastein formation from the thermodynamic standpoint. The thermodynamics of the plastein reaction is influenced solely by the properties of the peptides constituting the substrate, because the enzyme is added in an amount such that it cannot influence significantly the equilibrium position in the system. Comparatively few studies have been made on the thermodynamics of the peptide bond[89-96]. Unfortunately, owing to experimental difficulties, only the thermodynamic parameters of protected dipeptides and certain aminoacid derivatives have been investigated. It can be shown that the hydrolysis of a protected dipeptide is a satisfactory model, from the thermodynamic point of view, of the hydrolysis of a long peptide, which does not alter the ionisation constants of the terminal ionogenic groups. As will be shown in the Section devoted to the influence of the length of the substrate peptide on plastein formation, the synthesis and hydrolysis of peptides of precisely this length occur in the plastein reaction. It has been found that the change in the standard free energy in the hydrolysis of a long polypeptide to shorter peptides is negative and amounts to between -0.5 and 1.5 kcal mole$^{-1}$. If it is assumed that a mixture of peptides with an average molecular weight of 1000 is used as the substrate and that the concentration of the substrate in the reaction mixture is 50%, then the molar concentration of the substrate is 0.5 M. Taking into account the above standard free energies of hydrolysis, it

is easy to show by calculation that the reaction mixture will contain between 2 and 12% of the dimers of the initial peptides. Clearly the content of peptides with a high degree of polymerisation will be altogether negligible.

Thus the decrease of the free energy of the system on plastein formation cannot be due to the formation of new peptide linkages in concentrated solutions of protein hydrolysates. Consequently the main driving force of plastein formation is the increase of the entropy of the system, which occurs when the hydrophobic aminoacid chains, formed by a rearrangement of the aminoacid residues of the initial peptides, aggregate. Unfortunately a comparison of the aminoacid sequences of the peptides constituting the substrate and the plastein has not so far been made in any instance of plastein formation.

Thus, despite the fact that the rheological properties of plasteins have been investigated for a very limited number of specimens, the studies which have been carried out not only permit the determination of the possible ways of the practical employment of plasteins (see Section VI) but also definite conclusions concerning the driving forces in plastein formation and the structure of plastein molecules.

## IV. THE INFLUENCE OF VARIOUS FACTORS ON PLASTEIN FORMATION

For a correct assessment of the influence of various factors on plastein formation, it is necessary to know precisely whether the system is or is not in a state of thermodynamic equilibrium at the instant of measurement. It is still not clear whether the yields of the plastein reactions refer to equilibrium conditions. It has been shown in some studies that the process parameters cease to change with time after 6–8 h,[48] whilst the others it is stated that this requires 2–3 days[73]. No regularities have so far been observed as regards this problem. Nor is it known whether all the system parameters cease to change simultaneously, for example, parameters such as turbidity, molecular weight, amount of the given fraction precipitated from a 10% solution of trichloroacetic acid, etc. However, even if it were known that all the parameters cease to vary at a certain instant, there is still a possibility that the system is then at a local free energy minimum and that the activation barrier to further transformation is very high, i.e. the system is not in a state of equilibrium but is kinetically inhibited. For these reasons, the concept of the yield of the plastein reaction is better replaced by the concept of the efficiency of the plastein reaction, implying by this term the degree of plastein formation under certain specified conditions with respect to the given parameter.

### 1. Dependence of the Efficiency of Plastein Formation on the Characteristics of the Protein Employed

The efficiency of plastein formation has been investigated[97] as a function of the protein used to prepare the pepsin hydrolysate as the substrate. The efficiency of plastein formation was estimated from the amount of plastein precipitated in a 10% solution of trichloroacetic acid. The experiments were performed at a substrate concentration of 20% and a concentration of $\alpha$- chymotrypsin, used to synthesise the plastein, of 0.1%. The mixture was incubated for 24 h at 37° C and then a solution of trichloroacetic acid was added at a concentration such that the final concentration was 10%. Under these conditions, the degree of plastein formation from the hydrolysates of different proteins is as follows: 94% for soya-bean protein, 58% for cod protein, 61% for a 9 : 1 mixture of cod and soya-bean proteins, 54% for chlorella protein, 80% for gluten, 35% for casein, 65% for baker's yeast protein, and 93% for petroleum yeast protein.

As stated above, the formation of the water-insoluble fraction is one of the main driving forces in the plastein reaction. Hydrophilic substrates do not form plasteins in high yields, because the products of the plastein reaction are then soluble and are difficult to remove from the system[97,98]. Thus attempts to synthesise plasteins from a highly hydrophilic protein (gelatin) were unsuccessful[53,98]. On the other hand, if the hydrolysate is too hydrophobic, the product is readily precipitated before the plastein formation reaction reaches an advanced stage[63].

Table 9. Dependence of the efficiency of plastein formation on the molecular weight of the substrate[22].

| Molecular weight | Percentage content of fraction precipitated with 10% trichloroacetic acid after incubation | | Molecular weight | Percentage content of fraction precipitated with 10% trichloroacetic acid after incubation | |
|---|---|---|---|---|---|
| | with enzyme | without enzyme | | with enzyme | without enzyme |
| >1043 | 6.6 | 3.3 | 685 | 33.2 | 0.0 |
| 1043 | 23.0 | 0.0 | <685 | 0.0 | 0.0 |

It has been shown[99] that, knowing the yield of the plastein reaction with respect to the high-molecular-weight fraction of the hydrolysates of individual proteins, it is possible to predict the component ratio for which the yield of the plastein reaction with respect to the high-molecular-weight fraction will be a maximum. For this purpose the parameter $\beta/\alpha$ was introduced, $\beta$ being the degree of insolubility of the plastein, defined as the ratio of the nitrogen insoluble in 20% aqueous acetone solution to the overall nitrogen content in the plastein and $\alpha$ the yield of the plastein reaction, defined as the ratio of the amount of nitrogen precipitated in 10% trichloroacetic acid solution to the overall nitrogen content in the plastein.

It has been shown in relation to extensive experimental data that, whatever the substrates or their mixture, the plot of the yield of the plastein reaction with respect to its high-molecular-weight fraction against the parameter $\beta/\alpha$ is bell shaped with a mixmum at $\beta/\alpha \simeq 0.5$. The dependence of the parameter $\beta/\alpha$ on the ratio of the contents (by weight) of the two substrates is then linear. Thus the ratio of the substrates in the mixture for which the plastein reaction has a maximum yield with respect to the high-molecular-weight fraction can be calculated from the formula

$$x/y = \frac{0.5 - \beta/\alpha\,(x)}{\Delta\beta/\alpha},$$

where $x/y$ is the ratio of the weight fractions of the two substrates, $\beta/\alpha(x)$ the value of the parameter $\beta/\alpha$ for the substrate $x$, and $\Delta\beta/\alpha$ the difference between the values of the parameter $\beta/\alpha$ for the substrates $x$ and $y$.

Thus, when the parameter $\beta/\alpha$ is used, it is possible to select a mixture of substrates giving the maximum yield of the plastein reaction with respect to the high-molecular-weight fraction.

2. The Influence of the Structure of the Substrate on the Efficiency of Plastein Formation

We shall now consider the requirements which must be met by the peptides in the substrate in order that plastein formation should be successful to the maximum extent.

The efficiencies of the fractions of soya-bean hydrolysate of different molecular weight in the plastein reaction have been compared[22]. The initial concentration of the substrate was 35% and the ratio of the weight concentrations of subtilisin BPN' and the substrate was 0.01. The reaction mixture was incubated for 24 h at 37° C. The intensity of plastein formation was estimated from the amount of precipitated fraction in 10% trichloroacetic acid solution. The results of the study are presented in Table 9, which leads the conclusion that plastein formation takes place most effectively in the system containing peptides consisting of 5–8 aminoacid residues[5,22–24]. In addition, it was found that the plastein reaction proceeds most effectively when the degree of hydrolysis of the initial protein is such that 20% of the hydrolysate (by weight) is precipitated in 10% trichloroacetic acid solution[10,15,25]. The synthesis of plasteins and peptides from pure aminoacids was unsuccessful[26,27]. The fact that free aminoacids do not participate in the plastein reaction has been confirmed by numerous experiments, which have demonstrated the impossibility of enriching the plastein in aminoacids when the latter are introduced into the reaction mixture in a free state[52,94] and also by the fact that the plastein is formed more successfully from the non-dialysable fraction of the protein hydrolysate[52].

There exists a view that, with increase of the molecular weight of the substrate, the mechanism of plastein formation changes from polycondensation to transpeptidation[23]. One of the incomprehensible features of the plastein reaction is the nature of the change of the content of amino-nitrogen in the reaction mixture when plasteins are formed. It was noted that, in the process investigated, the amount of primary amino-groups can decrease[47,64], increase[25,62], remain unchanged[52,101], and pass through a minimum[23,32,68]. It is very likely that, when 5–8-membered peptides are used, a polycondensation reaction, accompanied by a decrease of the content of primary amino-groups in the reaction mixture, takes place.

Subsequently, after the attainment of a critical length of the peptides, mainly a rearrangement of the aminoacid residues occurs with formation of sparingly soluble peptides and the liberation of freeamino acids and low-molecular-weight peptides[23,73]. As a result of this, the content of primary amino-groups in the reaction mixture increases. It may be that the contradictory data concerning the changes in the content of amino-nitrogen on plastein formation can be accounted for by using substrates of different molecular weight.

The problem of which aminoacids should make up the peptides in order that plasteins can be formed from the latter is very interesting. In the previous section it was stated that proteins should be moderately hydrophobic in order to produce hydrolysates capable of giving rise to plasteins in a high yield. Plastein-active peptides were first identified by Determann and coworkers[102–104], who showed that the peptides forming the plastein under the influence of pepsin and α-chymotrypsin have the following structure:

H—Met—Thr—Leu—Leu—(Leu, Thr, Val)—Thr—Ser—Met—Leu—OH
H—Tyr—Ile—(Glu, Gly, Phe)—(Glu, Ile)—Phe—OH
H—Tyr—(Asp, Val)—Glu—Glu—Ile—OH
H—Ile—Asp—Val—Glu—Glu—Ile—OH

The structure of these peptides shows that the efficiency of plastein formation is determined primarily by the terminal aminoacid. This hypothesis was first put forward by Virtanen et al.[51].

In order to establish a relation between the structure of peptides and their ability to form plasteins, studies were performed using as substrates various peptides having a definite structure[24,28–30,105,106]. The plastein reaction was carried out with the aid of pepsin at pH 4.0 and chymotrypsin at pH 8.6. A 25–50% solution of the substrate, incubated with the enzyme at 37° for 24 h, was used. The high-molecular-weight fractions of the plasteins were separated by centrifugation or were precipitated with trichloroacetic acid. The plasteins obtained under the influence of pepsin have a molecular weight of ~2250 with an average degree of polymerisation of 2.5, which was established by the photometric determination of the amount of terminal aminoacid residues in the form of dinitrophenyl derivatives[30]. The authors explain the low degree of polymerisation by the poor solubility of the resulting plasteins. It was found that, under the above conditions, plasteins are formed by peptides having the following structure[29]:

H—L—Tyr—L—Leu—Gly—L—Glu—L—Phe—OH
H—L—Tyr—L—Leu—Gly—D—Glu—L—Phe—OH
H—L—Tyr—L—Leu—Gly—L—Val—L—Phe—OH
H—L—Tyr—L—Leu—Gly—L—Lys—L—Phe—OH
H—L—Tyr—L—Ile—Gly—L—Lys—L—Phe—OH
H—L—Tyr—L—Lys—Gly—L—Glu—L—Phe—OH
H—L—Tyr—L—Lys—Gly—L—Lys—L—Phe—OH
H—L—Tyr—L—Leu—Gly—L—Glu—L—Tyr—OH
H—L—Tyr—L—Leu—Gly—L—Glu($NH_2$)—L—Tyr—OH

On this basis, it was concluded that aminoacid residues located in the centre of the peptide chain have no influence on the plastein reaction catalysed by pepsin and chymotrypsin. It is also unimportaint whether the peptide is basic, neutral, or acid.

The influence of chain length and of the nature of *N*- and *C*-terminal aminoacid residues on the plastein reaction has been investigated[24]. For this purpose, the following peptides were synthesised:

H—Ala—Leu—Gly—Glu—Phe—OH
H—Tyr—Leu—Gly—Glu—Ala—OH
H—Ala—Leu—Gly—Glu—Ala—OH
H—Tyr—Leu—Ala—Glu—Glu—Phe—OH
H—Tyr—Leu—Glu—Phe—OH
H—Tyr—Glu—Phe—OH

It was found that, for a peptide to be capable of involvement in the plastein reaction catalysed by pepsin or chymotrypsin, it is necessary that it should have hydrophobic *C*-terminal aminoacids and should consist of at least four aminoacid residues.

Thus two main requirements must be met by the substrate of the plastein reaction when the plastein is synthesised with the aid of pepsin or chymotripsin: it must consist of peptides formed from 5–8 aminoacid residues and in addition the aminoacid residues at the ends of these peptides should be hydrophobic. The possible reason why the plastein reaction takes place most successfully with peptides of a definite length is as follows. According to the thermodynamics of the peptide linkage, the polycondensation of peptides is thermodynamically more favourable than the polycondensation of aminoacids[89–96]. However, in solutions of peptides at identical weight concentrations the concentration of amino- and carboxy-groups is lower the higher the molecular weight of the substrate. In solutions

of peptides consisting of 5–8 aminoacid residues there is probably, on the one hand, still a fairly high concentration of amino- and carboxy-groups and, on the other, the formation of a peptide linkage between such peptides is already more favourable than between aminoacids. The fact that plastein formation requires the presence of hydrophobic aminoacids at the ends of polypeptides can be accounted for by two causes. Firstly, the thermodynamics of the peptide linkage can be such that the bond formed by hydrophobic aminoacids is thermodynamically most favourable. One can hardly expect a large difference between the thermodynamic parameters of the bonds formed by different aminoacids[89-96]; at the same time, it is known that the majority of proteolytic enzymes exhibit a greater or smaller specificity with respect to hydrophobic aminoacids[107,112]. It is therefore most likely that the limitations imposed on the structure of plastein-active peptides are due to the specificity of the enzymes employed. This hypothesis is supported by the fact that trypsin, the enzyme which hydrolyses and synthesises solely bonds formed by the carboxy-groups of lysine and arginine[113], does not exhibit a plastein-forming activity[114]. Nor is it possible to obtain plasteins from hydrolysates produced under the influence of trypsin[115].

### 3. The Influence of Substrate Concentration on Plastein Formation

The dependence of the efficiency of plastein formation on substrate concentration has been studied[18] using different enzymes. Plasteins were synthesised from the pepsin hydrolysate of soya-bean protein at substrate concentrations of 5, 10, 20, 30, 40, and 50% and for enzyme and substrate concentration ratios (by weight) in the range 0.01–0.03. The reaction mixture was incubated for 24 h at 37°C. The following enzymes were used: chymotrypsin, pepsin, coronase, bioprase, and molsin. The pH of the reaction mixture was maintained at values indicated by Yamashita et al.[114]. The efficiency of plastein formation was estimated from the weight of the fraction precipitated with a 10% solution of trichloroacetic acid. It was shown that in all cases the yield of plastein has a maximum at a substrate concentration between 30 and 40%. At substrate concentrations less than 6–8%, the substrate hydrolyses[18,19].

Analogous results were obtained in studies[23,44] involving the formation of plastein under the influence of pepsin from a pepsin hydrolysate of zein and in studies[20,21] on the preparation of plastein under the influence of pepsin and papain from the pepsin and papain hydrolysates of egg protein. According to modern views on the mechanism of plastein formation, the conclusion that the optimum substrate concentration for plastein formation is 30–40% and that plastein formation does not occur at a substrate concentration below 6–8% is probably common to plasteins obtained from any proteins under the influence of any enzymes.

### 4. The Influence of the Enzyme and Its Concentration on the Efficiency of Plastein Formation

As already mentioned, a very large number of proteolytic enzymes of different origin have been used in the plastein reaction. The only feature which these enzymes have in common is that they are all endopeptidases. The efficiencies of plastein formation under the influence of various enzymes have been compared systematically[97]. The data compiled in Table 10 show that $\alpha$-chymotrypsin is most effective in the plastein reaction, while other enzymes are less effective. One should note that the efficiencies of plastein formation listed in Table 10 for a series of enzymes (for example papain and pepsin) conflict with the wide use of these enzymes for the preparation of plasteins.

**Table 10.** Comparative efficiencies* of various enzymes in the plastein reaction[97].

| Enzyme | Yield of plastein, % | Enzyme | Yield of plastein, % |
|---|---|---|---|
| Endopeptidases: | | Microbiol proteases: | |
| α-chymotrypsin | 95.0 | bioprase | 85.5 |
| bromelain | 29.0 | coronase | 52.1 |
| papain | 26.8 | molsin | 21.3 |
| pepsin | 20.0 | pronase | 36.3 |
| trypsin | 21.9 | prozime | 77.0 |
| Exopeptidases: | | rapidase | 20.0 |
| aspergillocarboxypeptidase | 20.0 | thermoase PC-10 | 32.3 |
| carboxypeptidase A | 26.5 | | 20.0 |
| leucine aminopeptidase | 22.1 | | |

*The plasteins were synthesised from the pepsin hydrolysate of soya-bean protein. The efficiency of plastein formation was estimated from the weight of the plastein fraction precipitated in 10% trichloroacetic acid solution.

The absence of plastein-forming activity in exopeptidases can be explained by the fact that the linkage between peptides is thermodynamically more favourable than the linkage between a peptide and an aminoacid. The different plastein-forming activities of different endopeptidases can be explained only by the fact that at the instant of the measurement the yield of the plastein reaction is still far from equilibrium, because the enzyme, acting as a catalyst, cannot influence the equilibrium position in the system. The reason for the absence of plastein-forming activity of trypsin was examined above.

The influence of enzyme concentration on the efficiency of plastein formation has been studied[20,31,33,47,53]. It was shown that, firstly, in the plastein reaction the enzyme concentration can be approximately the same as the hydrolysis of proteins – up to 1 wt.%, and secondly, that the efficiency of plastein formation depends only very slightly on the enzyme concentration. Thus with increase of the concentration of the enzyme (pepsin) from 0.1 to 8%, the amount of the plastein fraction derived from the pepsin hydrolysate of egg albumin and insoluble in the reaction mixture increases from 13.1 to only 33.3%.[53] In a study of the synthesis of plastein by this procedure[31] a relation was found between the weight of the insoluble fraction and the weight of the enzyme employed: $P = 13.9E^{0.213}$, where $P$ is the weight of the plastein fraction obtained and $E$ the weight of the enzyme employed. An analogous result was obtained in a study[47] where plastein formation from the pepsin hydrolysate of zein under the influence of pepsin was investigated. The efficiency of plastein formation was estimated in this instance from the decrease of the content of amino-nitrogen, determined by the copper complex method[116].

The influence of enzyme concentration on the rate of plastein formation was hardly considered. It has only been stated in one study[20] that the rate of the plastein reaction, determined as the increase with time of the amount of nitrogen precipitated from a 2.5% solution of trichloroacetic acid, is proportional to the square root of enzyme concentration.

Substances which inhibit the activity of the enzyme employed retard or altogether terminate plastein formation[20,25,78,117].

When the influence of enzymes on the plastein reaction is considered, the following factors must be taken into account. It is known that all enzyme preparations contain a certain amount of inactive protein; the concentration of enzymes in solution, found from their weight concentrations, does not therefore represent their true concentration (normality) in solution. In order to determine the normality of enzyme solutions, it is necessary to find the concentrations of their active centres. However, no studies have been made on plastein formation with enzymes of known normality.

### 5. Dependence of the Efficiency of Plastein Formation on the pH of the Reaction Mixture

The dependence of the efficiency of plastein formation on the pH of the reaction mixture has been studied[22,23,31-33] The dependence of the plastein reaction on the pH of the solution was first investigated for the plastein synthesised using pepsin from the pepsin hydrolysate of egg albumin[31]. The efficiency of plastein formation was determined from the weight of the filtered fraction. It was shown that in this case the insoluble plastein fraction is formed most effectively at pH 4.0. The same pH optimum was found for the plastein reaction catalysed by pepsin[23].

The pH dependence of the rate of plastein formation from a pepsin hydrolysate of soya-bean protein has been investigated for different enzymes[22,32,73]. The rate of plastein formation was determined from the increment in the fraction precipitated in a 10% solution of trichloroacetic acid along the linear section of the kinetic curve. It was shown that, whereas the optimum in the hydrolytic effect of the enzymes employed is in the pH range from 2 to 11, the optimum for plastein formation using the same enzymes is the pH range between 3 and 7 and for the majority of enzymes between 4 and 6. Thus it was found that the optimum pH for the hydrolysis of $\alpha$-chymotrypsin is 8.0 and that for plastein formation is 5.3. If the difference between the optimum pH values for plastein formation and hydrolysis is denoted by $\Delta$ pH then all the enzymes investigated can be divided into three groups: the pepsin type with $\Delta\,\mathrm{pH} > 0$, the chymotrypsin type with $\Delta\,\mathrm{pH} < 0$, and the papain type with $\mathrm{pH} = 0$. Trypsin, which does not exhibit a plastein-forming activity over the entire pH range, constitutes an exception. The values of $\Delta$ pH are as a rule greater for enzymes belonging to the chymotrypsin type.

However, there are data showing that chymotrypsin is most effective in the plastein reaction at pH 7.3.[57] The efficiency of plastein formation was estimated in this case from the weight of the filtered fraction. The cause of this discrepancy between the data of different workers remains obscure. Despite this, the fact that the dependence of the efficiency of plastein formation on pH does not coincide with the corresponding pH dependence of the hydrolysis of proteins for all the enzymes investigated leads to the conclusion that the ionic nature of the substrate is an important factor influencing the formation of plasteins.

### 6. The Influence of Temperature on Plastein Formation

The study of the influence of temperature on plastein formation showed that the weight of the fraction insoluble in the reaction mixture and in 10% trichloroacetic acid solution increases appreciably up to the enzyme denaturation temperature at 60–65°C [29,33,118,119]. The causes of the change in the yield of the plastein reaction when the temperature is altered are not entirely clear. It has been suggested[118] that it is due to the extremely low free energy of the synthesis of polypeptides in the plastein reaction. In the light of present knowledge about the mechanism of plastein formation and its thermodynamics, this explanation is hardly valid. The reason is most probably that an increase of temperature to 55–60°C entails an increase of the energy of the hydrophobic interactions[82,83] responsible for the formation of the gel in the plastein reaction, which leads to an increase of the amount of the fraction sparingly soluble in water.

### 7. The Influence of the Ionic Strength of the Solution on Plastein Formation

The ionic strength of the solution can influence plastein formation either by altering the activity of the enzyme employed or by affecting the solubility of plastein and desplacing thereby the equilibrium position in one direction or another. The importance of ionic strength in plastein formation is indicated by the finding that the possibility of the formation of gels has been demonstrated on treating a 4% solution of the pepsin hydrolysate of egg albumin at pH 7.3 with chymotrypsin, provided that the solution contains 26% NaCl.[49] As already stated, without the addition of NaCl, plasteins are not formed at this substrate concentration.

A detailed study of the influence of ionic strength on the rate of plastein formation has been carried out in relation to the formation of plastein from egg albumin obtained with the aid of $\alpha$-chymotrypsin and neutral bacterial protease resistant to the action of high salt concentration[78]. It has been found that different salts act on plastein formation in different ways at a concentration of 1 M. The strength of their influence increases with increase of the charge of the salt cation. The addition of sodium, potassium and lithium chlorides increases the rate of plastein formation by 10%, while the addition of iron, aluminium, and zinc chlorides to the reaction mixture reduces the rate of the plastein reaction by 80–95%. The dependence of the rate of plastein formation on ionic strength, established with the aid of NaCl when $\alpha$-chymotrypsin is used, is represented by a curve with two maxima of approximately equal height (20–30%) at ionic strengths of 0.1 and 0.8; after the second maximum, there is a sharp decrease of the rate of plastein formation. When a protease which does not lose its activity in concentrated solutions was used, it was shown that the rate of plastein formation increases with increasing ionic strength and that in 3 M NaCl solution it increases by a factor of 2 relative to the initial value.

These studies, combined with studies of the dependence of the activity $\alpha$-chymotrypsin on the solubility of the plasteins obtained in NaCl solutions at different concentrations, suggest that ionic strength acts on the rate of plastein formation via different mechanisms at different salt concentrations. At low salt concentrations the rate of plastein formation increases owing to the increase of the activity of the enzyme. At high salt concentrations plastein is salted out, which promotes an increase of the rate of its formation, but the enzyme can then lose its activity. If the enzyme does not lose its activity, the rate of the plastein reaction increases; if the enzyme is inactivated, the plastein is formed at a lower rate.

## V. INCORPORATION OF VARIOUS AMINOACIDS IN PLASTEINS

The problem of the possible alteration of the aminoacid composition of high-molecular-weight fraction of plasteins in a desired direction has been studied on a fairly wide scale[12,42,52,60,61,63,70,71,100,120-124]. The possibility of increasing the content of all the essential aminoacids in the high-molecular-weight fraction relative to their content in the initial protein was demonstrated. The enrichment can occur on both laboratory and semi-industrial scales[120]. It has been shown that free aminoacids are virtually not incorporated in plasteins[42,52,100,121]. For the successful introduction of a particular aminoacid into a plastein, it is necessary to use aminoacid derivatives. The data in Table 11 show that the amount of the high-molecular-weight fraction of plasteins does not change when different enzymes are used for their synthesis, but the menthionine content in the resulting plastein depends significantly on the enzyme employed. Evidently different methionine derivatives have significantly different abilities to increase the content of this aminoacid in a plastein. Methionine is not incorporated at all if the amino-group is protected by the acetyl group. The incorporation of methionine into a plastein takes place somewhat more successfully if methionineamide is used as the source of the aminoacid.

Table 11. Efficiency of plastein formation and of the incorporation of methionine into plasteins synthesised with the aid of various enzymes using different sources of methionine*.

| Enzyme | Yield of plastein reaction | Methionine content in the plastein fraction investigated (in wt.%) after addition of the following methionine derivatives into reaction mixture: | | | | |
|---|---|---|---|---|---|---|
| | | Met | AcMet | $MetNH_2$ | MetOEt | Met—Met |
| — | — | 1.15 | 1.44 | 1.57 | 2.10 | 1.50 |
| Ficin | 71.68 | 1.05 | 1.47 | 1.32 | — | — |
| Molsin | 68.30 | 1.18 | 1.53 | 2.09 | 3.87 | 2.70 |
| Nagarse | 75.00 | 1.20 | 1.22 | 3.12 | 7.22 | 5.10 |
| Papain | 78.20 | 1.15 | 1.32 | 1.43 | — | — |
| Pronase | 70.10 | 1.25 | 1.22 | 1.25 | 1.23 | 1.27 |
| — | — | | | | | |

* The yield of plastein was determined relative to the non-dialysable fraction of the total mixture by weight.

This shows that the amino-group of the incorporated aminoacid is directly involved in the reaction when methionine is incorporated into a plastein. The methionine content in the plastein increases appreciably when dimethionine and particularly the ethyl ester of methionine are used as the source of methionine. This shows that, although the carboxy-group of the aminoacid is not directly involved in the reaction, its preservation in an active form is essential for the successful incorporation of methionine in a plastein.

From the practical point of view, the possibility of enriching the high-molecular-weight fraction of plasteins in glutamic acid is of considerable interest[123]. Studies have been undertaken on the comparative efficiencies of various glutamic acid esters as a source of glutamic acid in the plastein reaction catalysed by papain[42]. The results of the investigations are presented in Table 12, which shows that the glutamic acid content in the high-molecular-weight plastein fraction can be greatly increased by employing the hydrophobic $\alpha,\gamma$-diethyl ester as the source of the aminoacid. The hydrophobic nature of the diethyl ester is almost the same as that of leucine[12].

Table 12. Comparative efficiencies of the employment of various glutamic acid esters as sources of glutamic acid in the plastein reaction catalysed by papain[12]*.

| L-Glutamic acid and its esters | Yield, wt.% | | Glutamic acid content, wt.% |
|---|---|---|---|
| | w.r.t. hydrolysate | w.r.t. substrate | |
| Glu-$\alpha,\gamma$-$OEt_2$ | 95.7 | 63.8 | 41.93 |
| Glu-$\alpha$-OEt | 54.3 | 36.3 | 32.18 |
| Glu-$\gamma$-OEt | 58.1 | 38.7 | 25.34 |
| Glu | 56.9 | 37.9 | 25.00 |
| — | 60.0 | 60.0 | 24.81 |

* Hydrolysate = pepsin hydrolysate of soya-bean protein; substrate = 2 : 1 mixture (by weight) of hydrolysate and glutamic acid derivative.

In order to investigate the enrichment in lysine of the plastein derived from the pepsin hydrolysate of gluten, synthesised with the aid of papain, a study was made of the influence of the ratio of the weight concentrations of the ethyl ester of lysine and the gluten hydrolysate on the effectiveness of the incorporation of lysine in the plastein. It was found that the maximum degree of incorporation of lysine is reached for a 1 : 2 ratio of the concentrations of the ethyl ester of lysine and the gluten hydrolysate[19]. Similar studies on the enrichment of plasteins in other aminoacids have not been made, but in most investigations the above ratio of the weight concentrations of the aminoacid ester and the protein hydrolysate was in fact used.

A study was made of the dependence of the rate and degree of incorporation of various aminoacids, added to the reaction mixture as aliphatic alcohol ethers of various structure, in plasteins whose formation was catalysed by papain[19,70]. It was found that, when use was made of derivatives of aminoacids with respect to which papain is non-specific (glycine, alanine), the amount of aminoacid ethyl ester incorporated in the plastein increases linearly with the plastein formation reaction time in the presence of the aminoacid ester, amounting to 20% of the total quantity of ester employed in the reaction after 6 h. When the ethyl esters of aminoacids to which papain is specific (L-$\alpha$-aminobutyric acid, norvaline, norleucine) are used, the incorporation of the aminoacids under virtually the same conditions terminates 2 h after the start of plastein formation; under these conditions, between 50 and 80% of the initial amount of the aminoacid ester is involved in the reaction. The degree of plastein formation after 2 h is only 50%. A distinct correlation has been found between the hydrophobic properties of the side chain of the aminoacids and the rate of their incorporation in the plastein when their ether esters are used. The degree of incorporation of glutamic acid, employed in the form of its diether ester, is the same as for norleucine. Aminoacids branched in the $\beta$-position (valine and isoleucine) constitute an exception. For a successful incorporation of the aminoacid, the alcohol with which it is esterified must have an unbranched structure. This requirement is particularly important when aminoacids with respect to

which papain is non-specific are incorporated. The incorporation of any aminoacid is faster the longer the aliphatic chain of the alcohol esterifying the aminoacid. Methionine is linked to the plastein polypeptide chains via peptide bonds[122] and this does not entail the formation of sections of chains consisting exclusively of methionine residues. D-Aminoacids are never incorporated in the plastein.

Thus the structure of the side chain of aminoacids influences primarily the degree and rate of their incorporation in the plastein. The structure of the alcohol esterifying the aminoacid has a smaller influence. The following remarks may be made concerning the mechanism of the incorporation of methionine. Analysis of the terminal amino-groups of the plastein molecules enriched in methionine showed that the methionine residue is located mainly close to the *C*-end of the polypeptide chain of the plastein molecule[61,122]. A three-stage mechanism of the catalysis, involving the formation of an acyl derivative of the enzyme, may be regarded as demonstrated for papain[125]. In this case the similarity of the distribution of methionine residues suggests that the incorporation of methionine in the polypeptide chain involves the formation of a peptidyl derivative of the enzyme, which undergoes nucleophilic attack by the amino-group of the esterified methionine with liberation of free papain and the elongation of the chain by one methionine residue. Subsequently the alcohol molecule is split off and the process can be repeated. Esterification of the carboxy-group promotes in this instance the enhancement of the nucleophilic properties of the methionine amino-group. Any modification of the carboxy-group, for example, the conversion of methionine into the amide of the formation of a dipeptide, has a similar influence on the reactivity of the methionine amino-group, but alcoholic groups are readily split off, which permits the repetition of the process. An alternative mechanism involves the formation of methionine-papain and nucleophilic attack by the peptide amino-group.

The first mechanism, involving the formation of a peptidyl derivative of the enzyme, is supported also by the distribution of the methionine residues preferentially at the *C*-end of the peptide chains and the absence of the incorporation of methionine in the chain when methionine derivatives with a protected amino-group are used as a source of methionine. The same mechanism of the addition of methionine is also supported by the finding that no free methionine, which would have been formed inevitably as a result of the hydrolysis of methionine-papain, has been

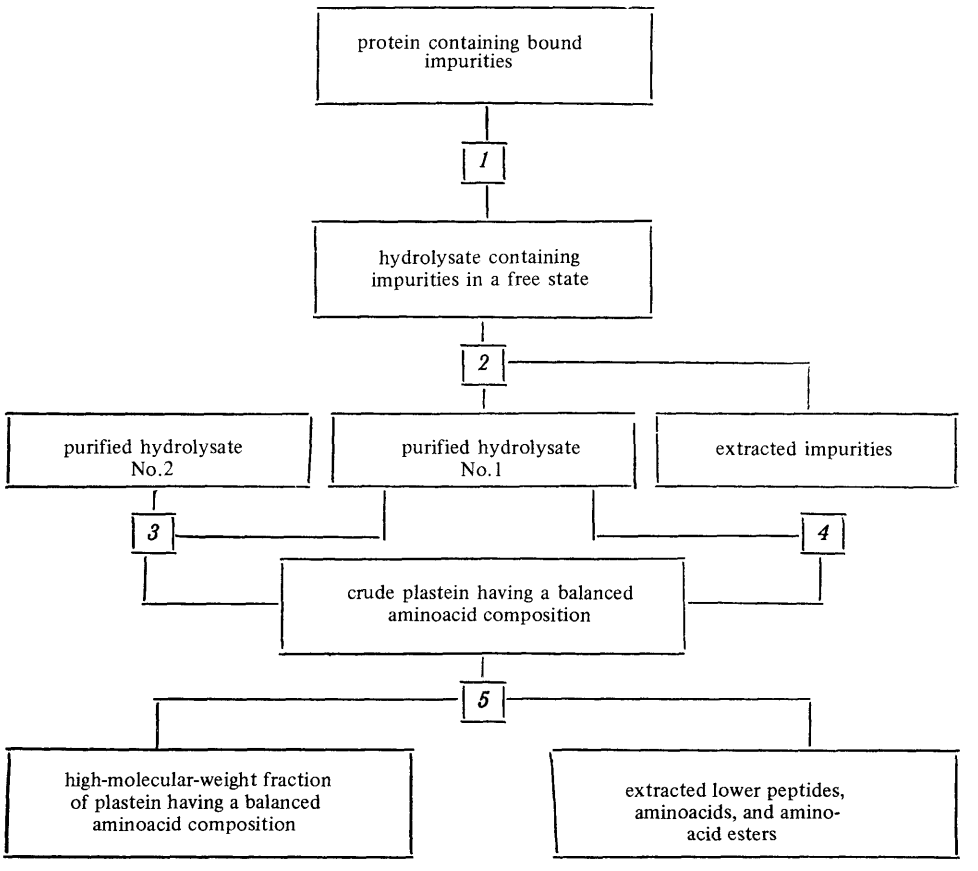



**Figure 1.** Synthesis of a plastein having a balanced amino-acid composition. Numerals 1 - 5 denote the following operation; 1) hydrolysis by endopeptidases; 2) treatment with organic solvents; 3) addition of endopeptidase; 4) addition of esters of essential aminoacids and endopeptidase; 5) treatment with aqueous ethanol.

found in the reaction mixture. If the reaction mechanism involving the formation of a peptidyl derivative of the enzyme is valid, the observed differences between the rates of incorporation of various aminoacids used in the form of their esters can be attributed to the differences between the binding of different parts of the aminoacid ester molecule to the subcentres $S_1$ for the aminoacid side chain and $S_2$ for the alcohol chain[125,128].

Studies on the mechanism of the incorporation of other aminoacids in plasteins under the influence of other enzymes have not been made. One can only postulate, on the basis of general information about the mechanism of the action of endopeptidases[107-111,113,125], that the mechanism of incorporation in such cases is analogous to that examined above. One should note that in many instances a mechanism involving the acylation of the enzyme by the aminoacid derivative is extremely probable. This is indicated by the appreciable incorporation in a plastein of phenylalanine and lysine when the latter are used in the form of ethyl esters with a protected amino-group[52,63,121].

In the enrichment of plasteins by aminoacids side reactions were noted solely in the enrichment of plasteins in lysine catalysed by papain[63]. An appreciable amount of lysine, dilysine, trilysine, and the ethyl ester of dilysine was detected in the reaction mixture only $1\frac{1}{2}$ h after the start of the reaction.

## VI. THE USE OF PLASTEINS IN NUTRITION

### 1. Purification of Proteins and the Preparation of Protein Products with the Required Aminoacid Composition

Numerous studies[19,42,52,60,61,63,70-72,100,120-122,129-132] have shown that protein products with a high food value can be obtained with the aid of the plastein reaction from proteins containing toxic and other undesirable impurities or an unbalanced aminoacid composition. The content of aminoacids in the plasteins obtained is close to ideality under these conditions[1,2]. A scheme for the process is illustrated in Fig. 1. Naturally, protein products with a balanced aminoacid composition can be obtained by simply mixing in definite proportions proteins which are unbalanced with respect to different aminoacids. However, such proteins are by no means always available in a pure form. It is much simpler to purify the hydrolysates than the initial proteins, because the impurities in proteins are incorporated in a network of polypeptide chains. The use of enzymatic protein hydrolysates in food is difficult, because they have a bitter taste and the employment of solid protein products is more customary for man. The main aim of the use of the plastein reaction in this instance is therefore to obtain with its aid protein-like substances from purified hydrolysates. Furthermore, it is naturally important that the enrichment should be by aminoacids synthesised chemically, the latter being introduced into the mixture in the form of ethyl esters.

Studies have shown that plasteins do not have toxic or other undesirable properties and that their nutrient properties are at any rate not inferior to those of the initial proteins and those of plasteins enriched in essential aminoacids are actually superior[72].

Protein-like products with a reduced content of any aminoacid, for example phenylalanine, can be obtained with the aid of the plastein reaction[124,129]. The use of such protein products is the only way of providing protein to persons suffering from phenylketonuria. A scheme for the preparation of plasteins with a low content of phenylalanine is present in Fig. 2. Successive hydrolysis with pepsin and pronase § was essential to ensure that aromatic aminoacids were present in the hydrolysate in a free state. It was found that it is possible to select gel filtration conditions under which aromatic aminoacids are removed from solution almost quantitatively. In order to restore in the resulting plasteins the required levels of tyrosine and tryptophan, the reaction mixture was treated, before plastein formation, with the ethyl esters of the above aminoacids. The subsequent operations involve the purification and more complete isolation of the plastein. As a result of the above process, the phenylalanine content is reduced from 4-5% to 0.05-0.20%, while the content of all the remaining essential aminoacids remains high.

### 2. Elimination of the Bitter Taste from Enzymatic Hydrolysates

As already mentioned, one of the reasons why enzymatic protein hydrolysates cannot be used in food is their bitter taste[97]. The conclusion that bitter peptides are formed in the enzymatic hydrolysis of proteins can probably be regarded as general for all proteins and all endopeptidases. Bitter peptides with various structures have been isolated from the pepsin hydrolysate of soya-bean protein[133-141]. A characteristic feature of these peptides is the presence of the leucine residue at the ends of the molecule, particularly at the C-end. The important role of hydrophobic terminal aminoacids in the generation of the bitter taste of peptides has been generally observed.

In order to eliminate the bitter taste of enzymatic protein hydrolysates, one can use various procedures. When protein hydrolysates are passed through a charcoal column, the bitter peptides are absorbed preferentially on the charcoal[142]. The bitter taste of enzymatic protein hydrolysate is reduced when the latter are treated with exopeptidases[135,138,139]. However, this method suffers from definite limitations, because, on treatment of the hydrolysates with exopeptidases, free aminoacids are liberated in considerable amounts, which can influence the food value of the hydrolysates.

The plastein reaction proved to be a convenient means for the elimination of the bitter taste from enzymatic protein hydrolysates. It has been found that, when the bitter hydrolysates are used as substrates of the plastein reaction, the resulting products are tasteless[59,97,133,143]. It has been shown that the disappearance of the bitter taste in the plastein reaction is associated with the formation of longer peptides in which the bitter peptides are constituents[73]. Enzymatic hydrolysis of plasteins results in the reappearance of the bitter taste.

The influence of an increased content of glutamic acid on the taste of enzymatic hydrolysates and plasteins is of interest. Plasteins enriched in glutamic acid have no taste, like ordinary plasteins. However, if plasteins with an increased glutamic acid content are hydrolysed by endopeptidases, the bitter taste does not reappear[42,60]. Such a hydrolysate contains peptides of three types: basic, neutral, and acid. The peptides of the first two types are bitter, while those of the third type have no taste. If the tasteless peptides are mixed with peptides of the first two types (jointly or separately), the bitter taste vanishes[42].

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§ Alkaline bacterial protease from *Streptomyces griseus*.

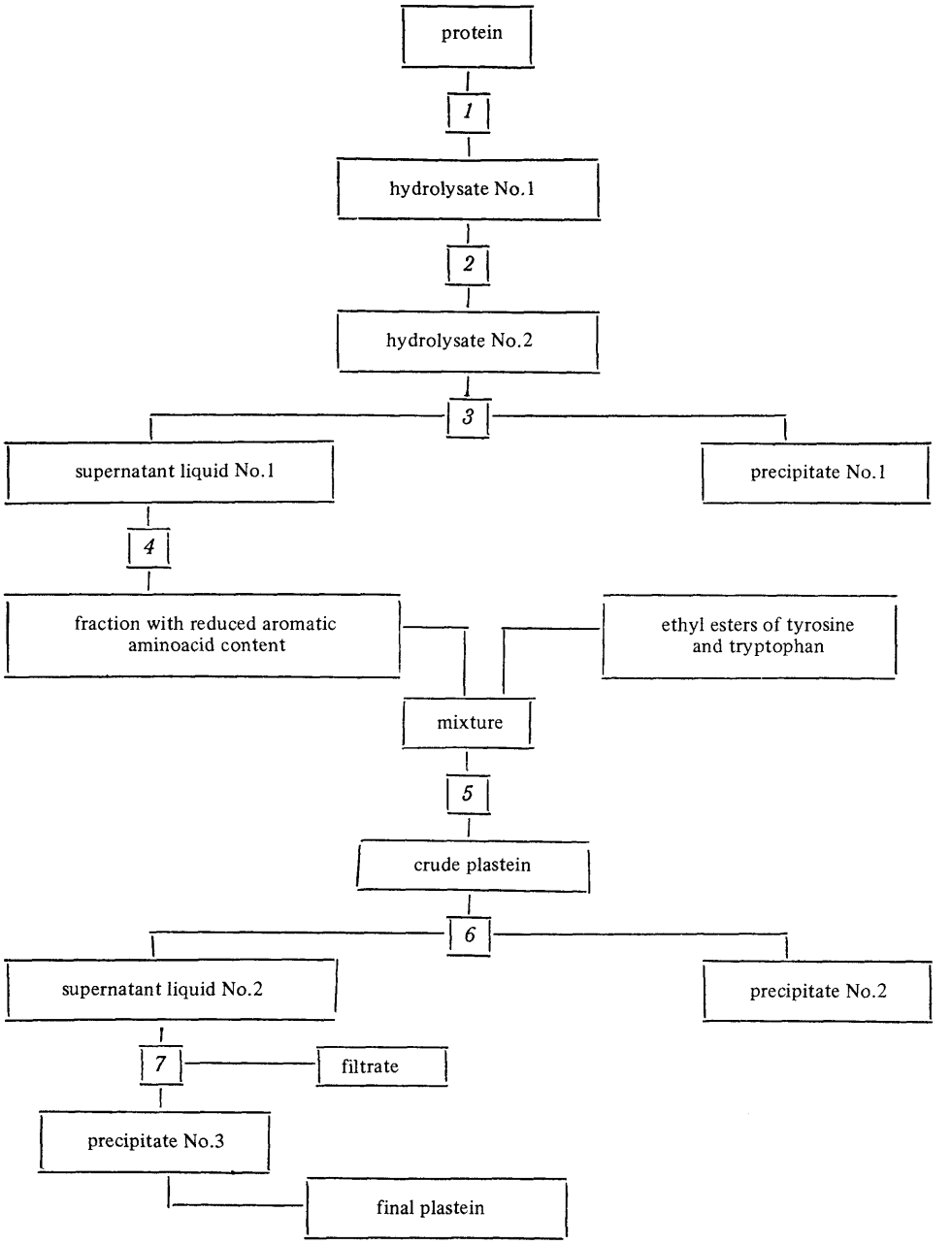



**Figure 2.** Synthesis of a plastein with a low phenylalanine content. Numerals 1–7 denote the following operations: 1) hydrolysis with pepsin; 2) hydrolysis with pronase; 3) centrifugation; 4) gel filtration; 5) synthesis with papain; 6) centrifugation; 7) ultrafiltration.

The principal component of the peptides of the first type is diglutamic acid. It has been found that diglutamic acid is capable of masking the bitter taste not only of peptides but also of other bitter substances such as $MgCl_2$, caffein, phenylthiourea, and brucine[130]. Consequently in this case the bitter taste disappears as a result of the masking activity of diglutamic acid and not as a result of the formation of new peptides.

Thus the plastein reaction can be useful not only for the preparation of protein-like substances with a high food value from proteins with an unbalanced aminoacid composition but also in the preparation of tasteless enzymatic protein hydrolysates from bitter hydrolysates.

3. Preparation of Protein Pastes and Gels with a High Food Value for Use as Fillers and as Products with an Independent Nutrient Value

The possibility of using plasteins in this way arises from their suitable rheological properties. The problem of the nutrient value of such protein pastes and gels was examined above.

4. Preparation of Mixtures of Peptides with a Particular Smell, Taste, and Other Characteristics for use as Food Additives and Flavours

As already mentioned, plasteins are usually tasteless. It has been shown that enzymatic hydrolysates of plasteins enriched in glutamic acid have the smell and taste of meat[42]. One cannot rule out the possibility that, under certain conditions, one can obtain plasteins or their hydrolysates with any other smell, or taste, but systematic studies on this problem have not been made.

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Summarising the studies carried out in the field of plastein formation, the following points must be noted. The principal practical applications of plasteins have now been outlined. It has been shown that with their aid one can use in food proteins derived from non-traditional sources and chemically synthesised aminoacids. However, the mechanism of the plastein reaction has so far been established only in a very broad outline. The determination of the mechanism of plastein formation is of interest not only from the theoretical but also from the practical point of view, because this can lead to new ways for the preparation and application of plasteins, which will increase the raw material base of the food industry.

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