

Action of Rennin and Pepsin on α -Casein: Paracasein and Soluble Products

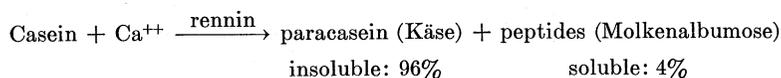
J. Cerbulis, J. H. Custer and C. A. Zittle

From the Eastern Regional Research Laboratory,¹ Philadelphia, Pennsylvania

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INTRODUCTION

Hammarsten (1) first observed the proteolytic action of the enzyme rennin on casein. Later investigations have been in general agreement on the reaction as formulated by Hammarsten (1):



The soluble fraction (peptides) contains a number of components (2-6). The ratio of soluble and insoluble reaction products varies some according to the reaction time, the precipitant used in separation (2, 6), and the preparation of α -casein (7). One of the major soluble components has been shown to be a glucopeptide (8). There have, however, been very few publications about the composition of the insoluble paracasein fraction.

Early conclusions (9) that the action of rennin and pepsin on casein is practically identical have been supported by recent studies (10-12). Others (4) have reported that during a 2-hr. reaction, there is some difference in split products.

The present investigation was undertaken to learn about the number and the nature of the reaction products, particularly whether minor components are present in the paracasein fraction. At the same time the action of the enzymes, rennin and pepsin, has been compared. The investigation is devoted principally to α -casein since this is the only individual component of casein showing the clotting reaction.

EXPERIMENTAL

Materials

Pepsin, crystalline. Commercial product.

Rennin, highly purified. Obtained through the courtesy of Dr. R. A. Sullivan,

¹ Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

National Dairy Research Laboratories, L. I., N. Y. This rennin had the same specific activity as crystalline rennin.

Whole Casein. Prepared by three-time, acid precipitation at pH 4.7 and washing with ethanol, acetone, and ether.

α -Casein. Prepared by the method of Hipp *et al.* (13) utilizing differential solubility in aqueous urea solutions. This product is relatively stable with calcium ions. A modification (7) of this procedure gave the calcium-sensitive product mentioned later.

Preparation of Dry Fractions

Precipitates were dried by washing with ethanol, acetone, and ether, and removing the solvent in a stream of air. Solutions were concentrated and dried by a rapid, large-surface vacuum technique (14) at from 30 to 35°.

Enzyme Reaction

Two per cent whole casein or α -casein solution was prepared by dissolving 10 g. of dry protein in a mixture of 56 ml. of 0.1 *N* NaOH and 440 ml. of distilled water, and the solution was adjusted to pH 6.4. Five milligrams of enzyme was dissolved in 5 ml. water and added to the casein solution at 30°C. The reaction was run for a definite time at 30°C. Previous experiments with casein solutions containing 15 mmoles CaCl₂/l. had established that the 5 mg. enzyme would give a clot in less than 1 min. (6). The enzyme reaction was stopped by placing the flask in a boiling water bath and stirring for 15 min. Then the flask was quickly cooled to room temperature and adjusted to pH 4.7 with 0.1 *N* HCl and centrifuged. Paracasein was dried as described above. The pH 4.7-soluble portion (peptides) was dried by a vacuum technique (see above).

Fractionation of Paracaseins

The procedure is outlined in Fig. 1. Details of the procedure are as follows: The dried isoelectric paracasein (10 g.) was extracted four times, each time with 150–200 ml. of the *n*-butanol–acetic acid–water (4:1:1; *v/v/v*) solvent system, by stirring for 15 min. and then centrifuging. The insoluble fraction was washed with ether and air-dried. Subsequently, this fraction was dissolved in dilute NaOH at pH 7.2 and precipitated at pH 4.7 with HCl, and the precipitate was dried as above. The supernatant at pH 4.7 was concentrated to dryness in a vacuum.

The solvent was removed from the 4:1:1-soluble fraction by vacuum distillation or by adding 10–12 vol. ether and centrifuging. For further fractionation it was dissolved in dilute NaOH at pH 7.2. Twenty-five per cent trichloroacetic acid (TCA) was added until a concentration of 2% was reached, and then centrifuged. The residual aqueous fraction was concentrated to dryness by vacuum distillation and washed again with ether.

Fractionation of Peptides

The procedure is outlined in Fig. 1. Peptide fraction was concentrated to dryness by vacuum distillation at 35°C. The extraction of dry peptide fraction with *n*-butanol–acetic acid–water (4:1:1; *v/v/v*) solvent was carried out in a similar manner to the extraction of paracasein. The solvent was removed from the 4:1:1-soluble fraction by vacuum distillation.

The 4:1:1-insoluble fraction of peptides was suspended in dilute NaOH at pH

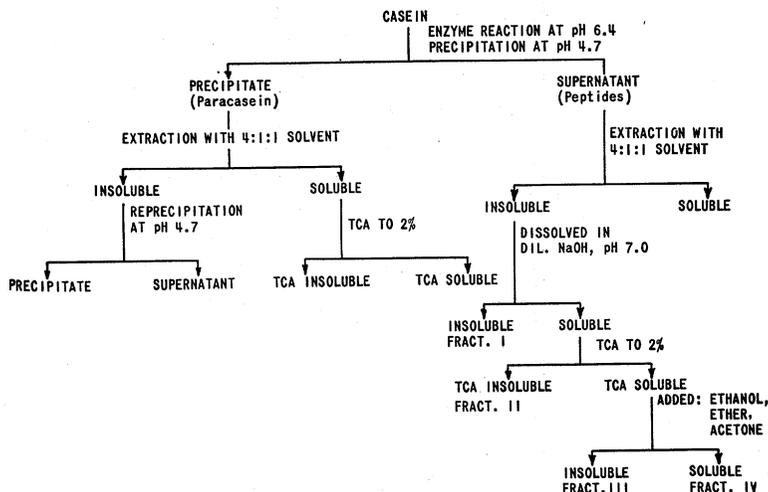


FIG. 1. The scheme of fractionation of paracaseins and peptides.

6.8–7.0, and an insoluble portion (Fraction I, Fig. 1) was removed by centrifuging. Twenty-five per cent TCA solution was added to the supernatant until a concentration of 2% was reached, and then centrifuged. Solids (Fraction II, Fig. 1) were washed and dried. Absolute ethanol (1.2 vol.), acetone (2 vol.), and ether (3 vol.) were added to the TCA-soluble fraction in order to precipitate peptides and to extract TCA (Fraction III, Fig. 1). These solids were dried. Further addition of ether or acetone to the soluble portion did not give any more precipitate. This solution was concentrated to the original volume by vacuum distillation, and then extracted four times with ether. The aqueous layer was concentrated to dryness by vacuum distillation, and the residue (Fraction IV, Fig. 1) was washed again with ether.

It was observed that all ether washings in all experiments, after evaporation of ether, left some yellowish fatlike residue, which on paper chromatography gave some spots with ninhydrin reagent for amino-containing substances, and with Oil Red O reagent for fats (15). These residues were not analyzed further.

Analytical Methods

The nitrogen contents of the proteins were determined using the Koch and McMeekin method (16). The phosphorus contents of the proteins were determined using Koch and McMeekin technique (16) for digesting the samples, and a modified Sumner method (17) for the color development.

Electrophoresis was carried out with a 1% solution at 1°C. in a Tiselius type electrophoresis apparatus for 3 hr.

Paper electrophoretic analyses of proteins and peptides were performed in a Durrum type electrophoresis cell (Spinco model R).² Electrophoresis was done at 5° for 16 hr. in a Veronal-HCl buffer, pH 8.6, ionic strength 0.075, on Whatman paper No. 1. Most of the experiments were done with 175 v., which gave a current flow of about

² Mention of commercial products does not imply recommendation by the U. S. Department of Agriculture over similar products not mentioned.

4.5 ma. Usually 0.01 ml. of a 6% solution of the substance under test was applied to the apex of the paper.

Paper chromatography was carried out using *n*-butanol-acetic acid-water-pyridine (30:6:24:20; *v/v/v/v*) as solvent.

Staining. The proteins generally were detected by staining the dried paper strips with bromophenol blue (18) and ninhydrin (19). Periodate-Schiff (20), and double oxidation toluidine blue (DOT) (21) stains were used to detect protein-carbohydrate components.

RESULTS

Paracaseins

Five-Minute Reaction. α -Paracaseins were prepared by the action of rennin and pepsin for 5 min. The distribution of N and P between precipitate and solution has been described in the previous paper (6). A 1:1 mixture of rennin and pepsin paracaseins presents the same symmetrical peak on electrophoresis by the Tiselius method at pH 8.6 as the individual paracaseins. The paper electrophoretic patterns of the two α -paracaseins are also identical (*b*, Fig. 2). In addition to a major component which migrates like α -casein, there is a minor component (*A*) which moves toward the negative pole. This minor component (*A*) and other minor components not visible at the concentration tested here, are concentrated by the extraction with 4:1:1 solvent and subsequent fractionation. Paper electrophoresis diagrams are shown later.

Twenty per cent by weight of the total α -paracasein was extracted with the 4:1:1 solvent (Table I). The reprecipitated 4:1:1-insoluble fraction is a homogeneous substance both by paper electrophoresis and by Tiselius electrophoresis at pH 8.6. The single symmetrical peak is in the α -casein region (*c*, Fig. 2). This component (*I*) gives a positive reaction with bromophenol blue, ninhydrin, periodate-Schiff, and DOT, just like α -casein itself.

The 4:1:1-soluble fraction on paper electrophoresis shows two bands (*d*, Fig. 2): one (*II*) in the α -casein region, which gives the same reactions as (*I*), only somewhat less intensive for carbohydrates; the other band, the *A* component mentioned above, with movement toward the negative electrode, is probably a low-molecular-weight component since it gives a reaction with ninhydrin only. Further fractionation of the 4:1:1-soluble fraction by TCA (Fig. 1) gives a TCA-insoluble fraction containing two components (*II* and *A*) (*d*, Fig. 2), and a TCA-soluble fraction. Paper electrophoresis shows (see *e*, Fig. 2) that the TCA-soluble fraction, when the casein is treated with rennin, contains two components (*B*, *C*); and with pepsin it contains three components (*B*, *C*, *D*). All were identified on the paper with the ninhydrin reagent. The relative position of these components is illustrated in *e* and *f*, Fig. 2 for a 3-hr. experiment with pepsin. These components (*B*, *C*, *D*) maintain the same relative position on paper

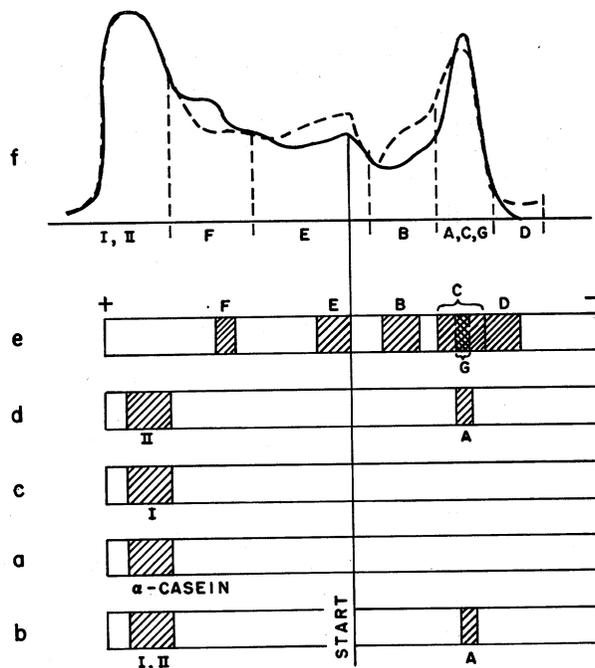


FIG. 2. Paper electrophoresis of α -casein and fractions insoluble at pH 4.7 (paracasein) obtained after the action of rennin and pepsin. *a* to *e* show schematic representations of bands obtained after electrophoresis and staining. α -Casein, components I, II, and G stain with both bromophenol blue and ninhydrin. Components A to F stain only with ninhydrin. *a*: α -casein; *b*: α -paracasein; *c*: 4:1:1-insoluble fraction; *d*: 4:1:1-soluble; also 4:1:1-soluble, TCA-insoluble fraction; *e*: 4:1:1-soluble, TCA-soluble fraction. *f* shows scanning curves obtained after the electrophoresis of paracaseins prepared after the action of rennin (solid line) and pepsin (dashed line) for 3 hr. The electrophoretic patterns were stained with ninhydrin. The large amount of background staining is due to the tailing of the I, II component, similar to α -casein.

electrophoresis, regardless of whether the reaction time is limited to 5 min. or extended to 3 hr.

Three-Hour Reaction. α -Paracaseins were prepared from α -casein by the action of rennin or pepsin for 3 hr. The products remaining in solution (peptides) at pH 4.7 after the 3-hr. action of the enzymes are, as per cent of total N or P: with rennin, 4.48% of N, 2.78% of P; with pepsin, 4.35% of N, 3.20% of P. This soluble fraction is relatively small in amount [compare (2, 4, 6)] because a calcium-sensitive α -casein, that is, a casein with less of the stabilizing fraction (7, 22), was used in this experiment. Comparison of α -casein preparations of different calcium sensitivities, however, has shown only quantitative differences and never qualitative. The soluble fraction (peptides) is discussed later. Fractionation of the paracasein was

TABLE I
Fractionation of Paracaseins
 Recovery of fractions by procedure given in Fig. 1

Paracaseins	Enzyme used	Reaction time	4:1:1 solvent			
			4:1:1 insoluble	4:1:1 soluble	TCA sol.	
					TCA insol.	TCA sol.
			(Total paracasein = 100)			
			%	%	%	%
α -Paracasein	Rennin or pepsin	5 min.	80.0	20.0	19.0	1.0
α -Paracasein	Rennin	3 hr.	80.0	20.0	14.5	5.5
α -Paracasein	Pepsin	3 hr.	70.0	30.0	11.0	19.0
Whole paracasein	Rennin or pepsin	5 min.	66.0	34.0	33.0	1.0
Whole paracasein	Rennin	30 min.	61.5	38.5	33.5	5.0

carried out as outlined in Fig. 1. Thirty per cent of α -paracasein prepared with pepsin, and 20% of α -paracasein prepared with rennin were soluble in 4:1:1 solvent (Table I). It was demonstrated by paper electrophoresis that the 4:1:1-insoluble fractions apparently contained only one component, and it had the same mobility as α -casein. Electrophoresis by the Tiselius method of these α -paracaseins, at pH 7.8, however, showed an irregular peak in the α -casein region with a slight shoulder on the slow side.

The 4:1:1-soluble fraction of both rennin and pepsin α -paracaseins showed paper electrophoretic patterns that differed in the amount of the various components (*f*, Fig. 2). In contrast to the 5-min. experiments, in the 3-hr. experiments there are sufficient of the minor components to show in the paper electrophoresis of this fraction. Fractionation by TCA gave a TCA-insoluble fraction, containing two components like those in the 5-min. experiments (*d*, Fig. 2). Both rennin and pepsin gave the same components. The paper electrophoretic patterns of TCA-soluble fractions (*e*, Fig. 2) were different for the α -paracaseins obtained with rennin and with pepsin. Component *B* and component *C* were released by both enzymes; the former was released much more slowly by rennin than by pepsin. Components *A* and *B* gave reactions only with ninhydrin. Component *C* gave a wide ninhydrin and carbohydrate band and a narrow bromophenol blue band (*G*) in the center, the same region where the band of component *A* in TCA-insoluble fraction can be found. Component *A*, however, does not give a reaction with bromophenol blue reagent. Components *D* and *E* were released by pepsin only, and the amounts were small. Component *F* is released by both enzymes, but the amounts were small. The amount

released by pepsin is less than that released by rennin (*f*, Fig. 2). The components *E* and *F* react only with ninhydrin.

The supernatant fraction from the pH 4.7 reprecipitation of the 4:1:1-insoluble paracaseins is small (about 1% of total). This fraction contains components which may be peptides (see next section) since on paper electrophoresis they have mobilities comparable to peptides in fraction III. Fraction III is soluble at pH 4.7, but some of this fraction may be carried down in the pH 4.7 precipitation of paracasein.

Fractionation of Peptides

Extraction with the 4:1:1 solvent also provides a useful fractionation for the soluble portion (peptides) arising from the action of rennin or pepsin on casein. The full scheme is outlined in Fig. 1. Analytical results showed that 20% of the N and 30% of the P were soluble in this solvent, when α -casein was treated with enzymes for 5 min. Further fractionation (see Fig. 1) of the 80% insoluble in the 4:1:1 solvent mixture gave the following distribution: fraction I, 1%; II, 5%; III, 69%; IV, 5%. When enzyme reaction was extended to 3 hr., 30% of the nitrogen was soluble in 4:1:1 solvent.

Peptide fractions were analyzed by paper electrophoresis and paper chromatography. Numerous solvent systems with paper chromatography were tried with little success. The most satisfactory was *n*-butanol-pyridine-acetic acid-water (30:20:6:24), but then only for the 4:1:1-soluble peptides. Paper electrophoresis had the broadest usefulness for the study of the peptides. Paper electrophoresis patterns of peptide fractions are shown in Fig. 3.

The various fractions had the following properties:

Fraction I of 4:1:1-Insoluble Peptides was grayish in color, insoluble in

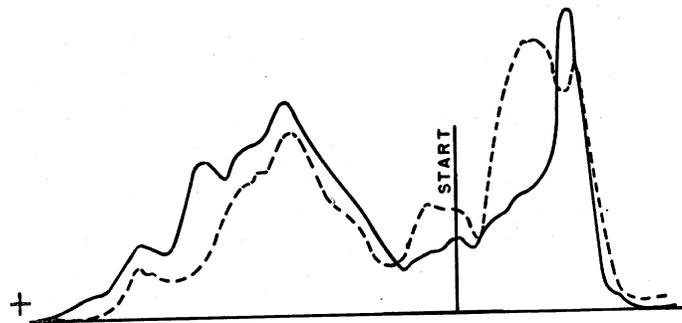


FIG. 3. Scanning curves of electrophoretic patterns of the total peptide fractions obtained by the action of rennin (solid line) and pepsin (dashed lines) for 3 hr. The electrophoretic patterns were stained with ninhydrin.

Veronal buffer pH 8.6, and relatively small in amount. It gave positive reaction with ninhydrin and bromophenol blue reagents, but no further analysis was carried out.

Fraction II of 4:1:1-Insoluble Peptides contained substance insoluble in 2% TCA. The paper electrophoretic patterns showed that this fraction contained a component which migrated identically with the major component of α -paracasein. The products of both rennin and pepsin gave positive reactions with ninhydrin and bromophenol blue reagents. Probably this represents the small amount of paracasein (major component) that is soluble at pH 4.7.

Fraction III of 4:1:1-Insoluble Peptides. This is the largest of the peptide fractions (60–75% of total peptides). The substances of this fraction, as is, are very soluble in water and are not dialyzable through cellophane membrane. In a large number of experiments with both rennin and pepsin preparations, paper electrophoresis showed that this fraction contained two or four components (Fig. 4). This variable number seemed to be related to the preparation of α -casein used. Such differences were not observed with the same preparation and different enzymes. Components 1 and 2 were found in all experiments, were observed in a very early stage of enzyme reaction, and reacted only with the ninhydrin reagent. Components 3 and 4 gave a positive reaction with the ninhydrin reagent and a weak bromophenol blue reaction. These components were obtained only with some preparations of α -casein and also then in very variable ratio to 1 and 2.

Components 1 and 2 migrated very closely together on paper electrophoresis patterns and in Tiselius type electrophoresis. Component 2 alone gave positive reactions with Schiff's and DOT reagents for carbohydrates. All attempts to separate 1 and 2 have been unsuccessful. This fraction (components 1 and 2) appears to be the glycomacropeptide described by Nitschmann *et al.* (8).

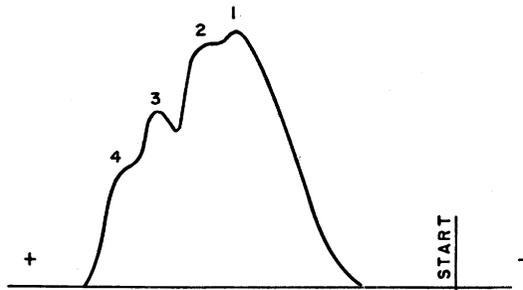


FIG. 4. Scanning curve of the electrophoretic pattern of fraction III of the peptides. Similar patterns are obtained from rennin or pepsin treatment of α -casein. The electrophoretic patterns were stained with ninhydrin.

Fraction IV of 4:1:1-Insoluble Peptides. This fraction was heterogeneous, and because of the small amount was not studied further.

The 4:1:1-Soluble Peptides. After 5 min. reaction time with either rennin or pepsin, this fraction contains only 20% of peptide nitrogen. After 3 hr. enzyme treatment, the fraction contains 30% of the peptide nitrogen.

The 4:1:1-soluble peptide fraction was analyzed by paper electrophoresis and by paper chromatography. All components gave a positive reaction only with the ninhydrin reagent. Both electrophoresis and chromatography showed that the 4:1:1-soluble fraction contained six to eight or more components. Some bands on paper electrophoresis patterns overlapped each other, and it was difficult to determine the exact number of components of this fraction. This was true also of paper chromatography. There were some differences between the split products obtained with rennin and pepsin. The split products of pepsin contained three components which were not found among the rennin split products. On the other hand, the split products of rennin contained a component not observed with pepsin.

DISCUSSION

Consideration of the results indicates that, in the main, action of rennin and pepsin on α -casein, under the conditions required for clot formation when Ca^{++} is present, leads to the same products. The major components of the paracasein fraction (components I and A) are the same, and fraction III, which represents 60–75% of the peptide fraction, is the same for both rennin and pepsin. There are minor differences (paracaseins TCA-soluble, and peptides 4:1:1-soluble) in the action of rennin and pepsin on casein, and since these differences are enhanced with prolonged action of the enzymes (Table I), they are probably due to a less specific proteolysis than that required for clot formation. Since pepsin gives several components (paracasein TCA-soluble, peptide 4:1:1-soluble) in 3 hr. that are not obtained with rennin, apparently pepsin has the broader specificity for the peptide bonds in casein that it can hydrolyze. Masayoshi (4) has reported that pepsin hydrolyzes casein more extensively than rennin when given sufficient time. Fish (23), comparing the action of rennin and pepsin on the B chain of insulin, had concluded that pepsin had the broader specificity.

Precipitation at pH 4.7, even though performed only once, is relatively selective for the separation of paracasein and peptide fractions. A small amount of the "peptide" fraction seems to be present in the 4:1:1-insoluble, pH 4.7-soluble fraction of paracasein. Also, fraction II of the peptides contains a small amount of material that may represent paracasein. Paracasein, even after three reprecipitations at pH 4.7, still contained minor components. The use of other precipitating conditions (TCA, Ca^{++}) will lead to paracasein and peptide fractions with different distribution of the

minor components (2, 8, 12). Calcium chloride was not used in the present research since analytical and fractionation procedures are simpler without calcium ion, and the calcium ion has no effect on the enzyme reaction (24).

The carbohydrate tests that were used were not sufficiently sensitive or specific to identify a carbohydrate-rich fraction. Positive tests were obtained with α -casein, the major component of paracasein, and the components 2 and *G* of the peptide fraction. Fractions 1 and 2 (Fig. 4) on the basis of the amount of this fraction probably corresponds to the glucopeptide described by Nitschmann *et al.* (8). These workers have postulated that this glucopeptide has an important role in stabilizing α -casein. When the glucopeptide is split off, clotting will occur in the presence of calcium ions.

Paper electrophoretic patterns and paper chromatography disclosed that the peptide fraction was very complex, containing eight or more components besides those in the paracasein fraction. Some of these components were only in traces, and it was impossible to give the exact number of peptides. Keller (3) has observed eight to ten components on paper chromatograms and on paper electrophoretic patterns, the major spot remaining at the origin.

Only a small portion of the peptide fraction was dialyzable, as was observed previously (6). It was found that the dialyzable peptides came only from the 4:1:1-soluble fraction, and this 4:1:1-soluble fraction was only 20% of the total peptides. Thus, the enzymes rennin and pepsin release mostly medium size or large peptides (6, 12).

SUMMARY

α -Paracasein, resulting from the action of rennin and pepsin on α -casein, and separated by precipitation at pH 4.7, is a heterogeneous substance. Fractionation and paper electrophoresis show it to contain a major component and several minor components, and Tiselius electrophoresis shows that even the major component is complex when the enzymes have acted for 3 hours. The soluble peptide fraction is very heterogeneous and contains 10-12 or more components. Extraction of both paracasein and peptide fractions with the 4:1:1 solvent mixture (*n*-butanol-acetic acid-water 4:1:1) is a useful procedure for separating fractions (soluble and insoluble) differing in composition. The glucopeptide described by Nitschmann *et al.* (8) was separated from other components by this means. The enzymes rennin and pepsin release the same major products, but there are differences in the minor products formed. The latter suggests that pepsin has the broader specificity.

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