

CLOTING OF CASEIN WITH PEPSIN : AMOUNT AND  
NATURE OF THE SOLUBLE PRODUCTS

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SUMMARY

The amounts of soluble products resulting from the action of pepsin on whole casein at pH 6.5 have been determined by precipitating the solutions with CaCl<sub>2</sub> (clot formation), and with 12% trichloroacetic acid, and by adjusting the pH to 4.7 with HCl.  $\alpha$ - and  $\beta$ -Caseins were studied in the same manner; with the latter, however, no clot was obtained. In general, 2 to 5% of the whole casein and  $\alpha$ -casein N became soluble by the action of pepsin, with the lower values obtained with trichloroacetic acid as the precipitant. Parallel increases in soluble P were obtained. Both paper and column chromatography showed that the soluble fractions contained a number of components, none of which were free amino acids or orthophosphate. Increases in the soluble fraction of  $\beta$ -casein indicated that considerable proteolysis had occurred. Most of the soluble fractions did not pass through a cellophane membrane.

When rennin acts on casein under suitable conditions to give a clot with calcium ions, about 4% of the casein is no longer precipitated at the isoelectric point or with trichloroacetic acid (1). The nature of this soluble fraction is important for an understanding of the clotting reaction (2). To initiate the present study of the clotting of casein, the same precipitating conditions (isoelectric, trichloroacetic acid) have been studied, with pepsin as the clotting agent. [Rennin and pepsin are similar in their clotting action and NPN release, although this is not true for all proteolytic enzymes (11)]. The amount of nitrogen remaining in solution when the calcium clot is removed has also been determined. In addition, the amount of casein phosphorus remaining in solution with the three precipitating conditions has been measured. These methods have been applied to whole casein, and to  $\alpha$ - and  $\beta$ -casein. The soluble fractions have also been subjected to dialysis and chromatography to determine their nature.

MATERIALS AND METHODS

*Casein.* The whole casein was precipitated from skim milk by acidification to pH 4.5 with *N* HCl. The precipitate was washed four times with water and twice dissolved and reprecipitated with acid (6). The casein was finally dried with absolute ethanol and ether.

*$\alpha$ -Casein and  $\beta$ -casein.* These caseins were prepared from whole casein by the urea fractionation procedure (7). All of the caseins were stored in a desiccator at a relative humidity that maintained the moisture content at 10.0%.

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The concentrations given are for moisture-free products. The solutions were prepared by dissolving the isoelectric caseins with the addition of sodium hydroxide.

*Paracasein.* The casein was clotted with pepsin, as described below. A suspension of the clot was adjusted to pH 4.7 and washed to reduce the calcium concentration. It was dissolved at about pH 7 and reprecipitated several times at pH 4.7. The paracasein was finally dried with absolute ethanol and ether.

*Pepsin.* The pepsin was a crystalline product obtained commercially (Armour).<sup>2</sup> It was dissolved in a small amount of water for use.

*Analytical methods.* The nitrogen was determined by the Nessler method after digestion with sulfuric acid. Phosphorus was estimated with the ammonium molybdate reagent after reduction with ferrous sulfate, on samples that had been digested with sulfuric acid and hydrogen peroxide.

*Chromatography.* For paper chromatography, the solvent systems most frequently used were *n*-butanol:water:acetic acid (4:1:5), benzene:water:propionic acid (25:25:62), *n*-butanol:acetic acid:water:pyridine (30:6:24:20), and water-saturated phenol. These were the most satisfactory. Numerous others were tried with little success. Both ascending and descending one-dimensional paper chromatography was used. For column chromatography the resin Amberlite IR-120(H+) was used. Successive fractions were eluted as described later.

*Color tests.* Peptides were spotted on the papers with the ninhydrin reagent. For inorganic phosphate, the ammonium molybdate reagent was used with reduction by stannous chloride (10). For organic phosphate, the same test was used but the ammonium molybdate reagent contained perchloric acid to release inorganic phosphate (3). In a few instances, spots were obtained with this reagent where there was no corresponding ninhydrin reaction, suggesting that cyclic phosphate-containing peptides might be present, or perhaps carbohydrate-phosphate compounds.

*Concentration of solutions.* The solutions were concentrated by a rapid, large-surface technique (4) at from 30 to 35° C.

*Dialysis.* The solutions were dialyzed for 72 hr. at 7° C. in a cellophane sac against four volumes of water. The water was changed every 24 hr.

*Action of pepsin on casein; methods of precipitation.* The formation of soluble products by the action of pepsin on casein was measured after precipitation of the casein in three different ways: (a) Precipitation of the isoelectric point by adjustment of the pH to 4.7 with 0.1 *N* HCl. (b) Precipitation by addition of concentrated trichloroacetic acid (TCA) to a concentration of 12%. [These procedures have been described by Alais *et al.* (1).] (c) Precipitation by clot formation with calcium chloride present (15 mM per liter). Unfortunately, with this procedure the soluble portion before pepsin treatment can not be determined directly as a control. To provide an indirect control, the soluble fraction was adjusted to pH 4.7 to precipitate any paracasein, and also

<sup>2</sup>Mention of commercial products in this article does not constitute a recommendation over similar products.

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the solubility of a purified paracasein under the clotting conditions was determined. With the concentration of whole casein used (2%), the calcium ion clots obtained with 15 mM of CaCl<sub>2</sub> per liter were flocculent. When the solutions also contained 5 mM of phosphate per liter, good clots were obtained, typical of those obtained with skimmilk. The gel-like clot given by phosphate, originally described by Hammarsten (5), is also obtained with  $\alpha$ -casein.

The pepsin was permitted to act on 2% whole casein at pH 6.5 and 30° C. In experiments with  $\alpha$ - and  $\beta$ -casein, the concentrations were 1%. Pepsin was added to whole casein in a ratio of 1:2,000 and to  $\alpha$ - and  $\beta$ -casein in a ratio of 1:1,000. Calcium ion, when present, was obtained by the addition of 15 mM of CaCl<sub>2</sub> per liter. The reactions were stopped at the end of 5 min., except in the time-course experiments, by the addition of TCA or by heating the mixture for 15 min. at 100° C. The mixtures were then cooled at 25° C. and the precipitations completed. The solutions were clarified by centrifuging, and subsequent determinations (N and P analysis, etc.) were performed on this soluble fraction. For chromatography, the solutions were concentrated by the method mentioned earlier.

#### RESULTS

In preliminary experiments, the time required for maximum formation of the specific soluble fraction by the action of pepsin on casein was found to be about 5 min. for the conditions employed. In confirmation of the findings of Alais *et al.* (1), when 12% TCA was the precipitant, slightly over 2% of the casein N became soluble in 5 min. (as shown in Table 1). There was no significant increase when the time was extended to 30 min. Alais *et al.* have shown (1), with the use of 2% TCA as the precipitant, that a nonspecific hydrolysis occurs, as well, which continues to increase beyond 5 min.

In Table 1 are shown also the amounts of soluble fraction obtained with precipitation at the isoelectric point of pH 4.7, and with CaCl<sub>2</sub> alone as the precipitating agent without changing the pH (6.5). For the latter, the solubility without pepsin can not be obtained directly, but adjustment of the soluble fraction to pH 4.7 shows that very little is precipitated which might be casein. An estimate of the inherent solubility of paracasein was made with purified paracasein precipitated under the same conditions (CaCl<sub>2</sub>, pH 6.5) (Table 1). Apparently, only about one-quarter of the soluble fraction can be paracasein. After correcting for this amount, it is found that when CaCl<sub>2</sub> alone is the precipitant, about 5% of the total N is made soluble by the action of pepsin. With the other precipitating conditions, the soluble fraction arising from the action of pepsin is obtained by subtracting the no pepsin value from the pepsin value. With precipitation at pH 4.7, the soluble fraction is 2.5%; with 12% TCA, only 1.7%. In general, parallel amounts of soluble P are obtained. With  $\alpha$ -casein the respective amounts of soluble N are 3.5, 6.0, and 1.7%.  $\beta$ -Casein gave no clot with pepsin, but considerable proteolysis was evident from the 20% soluble at pH 4.7, and 3.2% soluble in 12% TCA.

TABLE 1

*Increase in the soluble fraction (N and P) of whole casein, and  $\alpha$ - and  $\beta$ -casein after treatment with pepsin at pH 6.5, determined with three precipitating conditions<sup>a</sup>*

Precipitating conditions	Casein preparation	Treatment	Soluble fraction (casein = 100), %		Dialyzable (soluble = 100), %	
			Nitrogen	Phosphorus	Nitrogen	Phosphorus
Precipitated 12% TCA, CaCl <sub>2</sub> present	Whole	Pepsin	2.2	4.1		
Precipitated 12% TCA, CaCl <sub>2</sub> present	Whole	No pepsin	0.54	0.98		
Precipitated 12% TCA, no CaCl <sub>2</sub>	Whole	No pepsin	0.23	0.98		
Precipitated pH 4.7, no CaCl <sub>2</sub>	Whole	Pepsin	3.2	3.1	25.9	38.3
Precipitated pH 4.7, no CaCl <sub>2</sub>	Whole	No pepsin	0.72	1.55	17.2	17.9
Precipitated pH 4.7, CaCl <sub>2</sub> present	Whole	No pepsin	1.63	2.3	9.5	16.4
CaCl <sub>2</sub> , centrifuged pH 6.5	Whole	Pepsin	6.5	8.0	8.0	13.0
Soluble above, not precipitated pH 4.7	Whole	Pepsin	5.8	5.7	5.4	11.6
CaCl <sub>2</sub> , centrifuged pH 6.5	Paracasein	.....	1.7	2.1		
Precipitated 12% TCA, no CaCl <sub>2</sub>	$\alpha$ -Casein	Pepsin	3.7	5.9		
Precipitated 12% TCA, no CaCl <sub>2</sub>	$\alpha$ -Casein	No pepsin	2.0	4.5		
Precipitated pH 4.7, CaCl <sub>2</sub> present	$\alpha$ -Casein	Pepsin	3.8	5.6		
Precipitated pH 4.7, no CaCl <sub>2</sub>	$\alpha$ -Casein	Pepsin	7.1	8.2		
Precipitated pH 4.7, no CaCl <sub>2</sub>	$\alpha$ -Casein	No pepsin	0.8	4.1		
CaCl <sub>2</sub> , centrifuged pH 6.5	$\alpha$ -Casein	Pepsin	3.8	5.6		
Soluble above, not precipitated pH 4.7	$\alpha$ -Casein	Pepsin	3.5	5.2	17.6	34.9
Precipitated 12% TCA, no CaCl <sub>2</sub>	$\beta$ -Casein	Pepsin	4.0	5.5		
Precipitated 12% TCA, no CaCl <sub>2</sub>	$\beta$ -Casein	No pepsin	0.8	1.57		
Precipitated pH 4.7, no CaCl <sub>2</sub>	$\beta$ -Casein	Pepsin	21.6	8.75		
Precipitated pH 4.7, no CaCl <sub>2</sub>	$\beta$ -Casein	No pepsin	1.28	1.46		
Precipitated pH 4.7, CaCl <sub>2</sub> present	$\beta$ -Casein	Pepsin	11.6	12.7	27.8	11.9

<sup>a</sup> The amount of each fraction dialyzing through a cellophane membrane is also shown.

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Several of the soluble fractions were subjected to dialysis. The results (Table 1) show that most of the soluble fraction remained within the dialysis sac. This was the case even when the dialysis was carried on for several days.

The soluble fraction from a calcium chloride clot was concentrated and subjected to paper chromatography. By testing with several solvent systems, the fraction was found to be quite complex, consisting of at least ten components. The relative amounts of these components were not determined. None of the components were free amino acids or free phosphoric acid, judged by  $R_F$  values and specific color tests. Further, the fraction exhibited poor chromatographic behavior; some of the material remained at the starting point and strong tailing occurred, suggesting that some of the components were of rather high molecular weight.

A similar soluble fraction (96 mg. of N, 6.4 mg. of P) was separated into acidic, basic, and neutral fractions by chromatography on a 1 by 20 in. column of Amberlite IR-120(H+). The acidic fraction (17% of initial N and P) was in the filtrate and this was increased by about 20% by elution with 0.01 *N* HCl, the neutral fraction (52% of N, 38% of P) was eluted with 1 *N*  $\text{NH}_4\text{OH}$ , and the basic fraction (6% of N, 2.5% of P) with 1 *N* NaOH. Additional N and P were obtained in a P-rich compound (1.2% of initial N, 20% of P) which crystallized in the resin. The acidic fraction was hydrolyzed with hydrochloric acid. Paper chromatography of the hydrolyzed acidic fraction showed that there were 15 or more amino acids present, providing additional evidence that these components were of relatively high molecular weight.

#### DISCUSSION

Study of the action of pepsin on casein under the conditions required to give a clot with calcium chloride shows that 1.7% became soluble in 12% TCA, and 2.5% no longer precipitated at pH 4.7. In the case of  $\alpha$ -casein, the respective values were 1.7 and 6.3%. These values are of the same magnitude as values reported by Nitschmann and coworkers for the action of pepsin (11) and rennin (1) on whole casein and  $\alpha$ -casein (13). These workers also observed that the minimum amount of soluble fraction was obtained with 12% TCA. The present studies have, in addition, shown that the decrease in solubility on formation of paracasein is such that  $\text{CaCl}_2$  precipitation at pH 6.5 is feasible for removing the paracasein. The portion remaining in solution for whole casein and  $\alpha$ -casein is 5.0 and 3.5%, respectively, about the same as that remaining in solution at the isoelectric point, pH 4.7.

The release of P during the clotting of casein has not hitherto been studied systematically, but the present studies show that the solubilization of P parallels that of N. None of it is inorganic, judged by spot test. This parallel release of N and P is surprising, in view of the probably uneven distribution of phosphate-binding sites in the casein. The parallel in these values does not necessarily mean that a portion of the casein is completely hydrolyzed, but may be entirely coincidental. Since the present studies were completed, Nitschmann

*et al.* (14) have reported that clotting of casein leads to the appearance of an unusual protein-polysaccharide component in the soluble fraction. This component contains 11.4% N and 0.57% P, or a molar ratio of 44, very close to the value of 41 for whole casein.

Although, in general, soluble N and P values were in parallel, when TCA was the precipitant the apparent solubility of P exceeded that of N. Lability of phosphate ester bonds to TCA was considered a possibility. However, in experiments entailing more prolonged exposure to TCA there was no increase in the soluble P. The inclusion of calcium chloride in experiments comparing the different precipitating conditions (Table 1) led to variable results; in some instances the soluble fraction was increased, in others decreased. A study of the action of rennin on casein (8) indicated that the soluble fraction was 34 to 120% greater for 16 than for 12 mM of  $\text{CaCl}_2$  per liter.

Study of the soluble fraction by chromatography showed that it consists of ten or more components. A high molecular weight for some of the fractions is suggested by retention within a cellophane dialysis sac, and also by the presence of many amino acids in these components, suggesting that a fairly large polypeptide must be represented. Polymerization of small peptides into larger peptides during manipulation (plastein formation) is a possibility, but the presence of the large number of amino acids indicates that if this did occur, the resulting polymer could not have arisen from a single peptide. Results as a whole suggest that the soluble fraction arises from the rapid hydrolysis by pepsin or rennin of a number of susceptible bonds, giving rise to a number of relatively large peptides. The specificity of pepsin (15) suggests that the split will occur adjacent to phenylalanine or tyrosine. Wissmann and Nitschmann have observed (17) that phenylalanine appears as a new terminal group during the action of rennin on  $\alpha$ -casein.

Alais *et al.* (1) had previously concluded that the NPN fraction arising from the action of rennin consisted of more than one peptide. By varying the TCA concentration, a fractional precipitation is achieved (12% TCA—1.5 to 2% NPN; 2% TCA—4% NPN). By means of chromatography and electrophoresis on paper, the fraction soluble in 12% TCA appeared to be principally a single substance, whereas the 2% TCA soluble portion contained several components (1). Keller (9) observed about eight peptides on chromatography of the fraction soluble at pH 4.7, obtained by the action of rennin on whole casein and  $\alpha$ -casein. Much of this fraction, too, remained at the starting point.

The proteolysis of  $\beta$ -casein observed in the present study is considerably greater than the approximate 0.5% in a period of 5 min. observed by Nitschmann and Keller (13) with 12% TCA as the precipitant. In the same time, the specific proteolysis of  $\alpha$ -casein attained 1.6% (13). The casein preparations, however, show variability; in this same study, for example, Nitschmann and Keller had one sample of  $\alpha$ -casein become soluble to the extent of 3%. This variability might account for the divergent results with  $\beta$ -casein. Because of the nonspecific hydrolysis of  $\beta$ -casein, there would appear to be advantages in using

$\alpha$ -casein rather than whole casein in studying the clotting reaction, for fewer components would be expected in the soluble fraction than in whole casein. Further, with minimum clotting time these components would represent principally those arising from the specific split leading to clotting.

The protein-polysaccharide compound reported (14) to be released by the clotting of casein is nondialyzable, with a molecular weight of from 6,000 to 8,000. It is strongly hydrophilic, containing not only 40% of polysaccharide but large amounts of serine and threonine. It is this hydrophilic property which is considered to bestow on natural casein its solubility in the presence of calcium ions.

The recent studies of Waugh and von Hippel (16) and McMeekin, Groves, and Hipp (12) leave no doubt that the  $\alpha$ -electrophoretic component of casein is inhomogeneous. However, the amount of these new components [kappa-casein, for example, is reported (16) to be 15% of whole casein] makes it unlikely that they represent the protective colloid in natural casein which is split and solubilized during the clotting reaction. The recent report of Nitschmann *et al.* (14) appears to provide a more plausible explanation for the clotting of casein. The 1.5 to 2.0% of casein which becomes soluble during the clotting reaction is split from a major component of  $\alpha$ -casein, and the unusual, strongly hydrophilic properties of the split fraction bestow on natural casein its solubility in the presence of calcium ions. The effect of rennin on kappa-casein (16) suggests that this fraction of  $\alpha$ -casein may have the hydrophilic protein-polysaccharide bound to it.

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