Effect of a Proteolytic Enzyme Preparation (Alcalase) on Whey Proteins[1]

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ABSTRACT

When acid whey samples were adjusted to pH 7.5 and incubated with a commercial proteolytic enzyme preparation (alcalase), the turbidity of the solution gradually increased until a soft coagulum, composed of small-sized aggregates, formed after about 1 h. Under the same conditions, sweet whey produced no coagulated material unless $CaCl_2$ was added. Aggregate formation was enhanced by $CaCl_2$ addition in both wheys, but especially in acid whey. However, maximum aggregate formation in terms of protein recovery was about 28%. The precipitated material obtained with various amounts of Ca^{++} in both sweet and acid wheys resolved into two fractions when it was subjected to nondissociating gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis and HPLC studies showed that the polymerized product consisted of protein fragments having an approximate molecular weight of 7000 to 10,000. A possible mechanism for enzyme-induced aggregation of these peptides is presented.

INTRODUCTION

In a short communication, Mandhare et al. (5) presented preliminary data on removal of protein from sweet (rennet) whey by treatment with proteolytic enzymes and organic or inorganic salts as coagents. The objective of our experiments was to repeat the process on both sweet and acid wheys with a commercial proteolytic enzyme preparation (alcalase) and to gain an insight into the removal mechanism.

MATERIALS AND METHODS

Whey Preparation

Pasteurized skim milk was used for whey preparation. Casein was precipitated by acidification with HCl to pH 4.6 (acid whey) or by enzymatic treatment with a commercial rennet preparation (sweet whey). The curd was then removed by pressing and filtration. Sodium azide (.1 g/L) was added to the whey to avoid spontaneous fermentation during the experiments.

Alcalase Treatment of Whey Samples

Each whey sample was adjusted to pH 7.5 with 2 N NaOH. Then .38 AU (Anson Units)/L of alcalase .6 L (Novo Industri A/S, Bagsvaerd, DK), in liquid form suitable for food use, were added. The solution was incubated in a water bath at 37°C without shaking. During incubation, turibidity of the solution gradually increased. At the end of incubation (about 1 h), the coagulated material was collected by centrifugation at 1500 × *g* for 10 min. The precipitate was resuspended in the minimal amount of distilled water, and the resulting suspension was acidified to pH 3.5 with HCl to inactivate traces of enzyme activity, followed by freeze drying. The freeze-dried material was used for the characterization experiments. Whey samples were incubated in 100-ml beakers when the final volume was 50 ml or in 500-ml beakers for larger volumes. Control mixtures were made up with the enzyme solution in boiling inactivated water for 5 min.

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Electrophoresis.

The freeze-dried samples (50 mg) were dissolved in 5 ml Tris-glycine buffer (pH 8.3), and aliquots were subjected to nonreducing PAGE in 7.5% gels (4). For SDS-PAGE, the freeze-dried samples (50 mg) were dissolved in 5 ml of .0625 *M* Tris-HCl, pH 6.8, containing 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS (7), and incubated for 2 h at 37°C before use. Aliquots were electrophoresed in polyacrylamide gradient (7.5-15%) slab gels (.1 × 24 × 30 cm) at 100 V until the marker dye (Bromophenol blue) was 1 cm from the anode end of the slab (about 8 h). Following electrophoresis, the gels were stained for 2 h with .25% Coomassie brilliant blue R-250 and destained with 25% methanol containing 7.5% acetic acid (8). Protein bands were identified by the following standard proteins: IgG, bovine serum albumin (BSA), β-lactoglobulin (β-lg), and α-lactalbumin (α-la), all obtained from Sigma Chemical Co. (St. Louis, MO).

High Performance Liquid Chromatography

High performance liquid chromatography was performed with a Beckman apparatus (Beckman Instruments, Fullerton, CA) equipped with a TSK 3000 SW (7.5 × 600 mm) size exclusion column, an absorbance detector (Model 163), and a computing integrator. Chromatographic conditions (according to the manufacturers' suggestion) were: mobile phase, potassium-phosphate .1 *M* pH 7 containing .3 *M* NaCl; injection volume, 20 μl; flow rate, 1 ml·min^{-1}; detector wavelength 214 nm, isocratic conditions. To remove traces of insoluble material, the sample solutions were centrifuged for 10 min at 1000 × *g*. Afterward, they were passed through .45-μm (Amicon) microfilters prior to a run.

Chemical Analyses

Total N was determined by semi-micro-Kjeldahl procedure. Protein was calculated as the difference between total N (TN) and N soluble in 15% TCA (NPN). Total protein was expressed as (TN − NPN) × 6.38. Total Ca was evaluated by atomic absorption following the method proposed by Brule et al. (2). Ionic Ca was determined by a direct electrometric method using Ca-sensitive electrode (F 2112 Ca Selectrode Radiometer). For simplicity, the precipitated material is reported directly as a percentage of initial whey protein.

RESULTS AND DISCUSSION

In a preliminary series of experiments, sweet and acid whey samples were subjected to enzymatic treatment as specified in the Materials and Methods section. Furthermore, a test was carried out after adding anhydrous $CaCl_2$ as a coagent (5). This salt was added in suitable proportion to give the appropriate amount of ionic Ca. Composition of initial whey samples is reported in Table 1. The amount of precipitate produced, in terms of protein recovery, under various experimental conditions is reported in Table 2. Comparison of data in Table 2 suggests that precipitate formation is enhanced by the addition of Ca ions. This also suggests a reason for the difference in precipitate obtained with the two samples: the acid whey, which gives the highest yields, has a much higher Ca^{++} content than the sweet whey sample. Coagulation was not observed in the control mixtures, although those mixtures coming from acid whey samples produced a thin layer of sediment during incubation.

In a second series of experiments, acid whey samples were treated under the same conditions as the previous ones but with the addition of

TABLE 1. First series: composition of sweet and acid whey samples.

Whey	pH	Titratable acidity	Total protein	NPN	Ionic Ca
		(°SH)[1]	(%)		(ppm)
Sweet	6.65	2.2	.64	.23	115
Acid	4.75	11.0	.57	.20	1870

[1] °SH = Soxhlet Henkel degrees.

TABLE 2. First series: precipitate formation in terms of protein recovery from sweet and acid whey samples.

Sample description	Protein recovery
	(%)
From sweet whey	
Control: whey + heat-inactivated enzyme	. . .
Whey + enzyme	. . .
Whey + 1350 ppm Ca^{++} + enzyme	7.7
From acid whey	
Control: whey + heat-inactivated enzyme	7.0
Whey + enzyme	15.6
Whey + 350 ppm Ca^{++} + enzyme	25.3

increasing quantities of $CaCl_2$. The composition of whey samples is reported in Table 3. The results of the enzymatic precipitation trials are in Table 4.

Addition of Ca increased precipitate formation in both samples, but expecially in acid whey. Relationship between total Ca content and percent yield was linear (Figure 1). Extrapolation of the experimental curve toward the x-axis shows that a minimum of about 1400 ppm of Ca was required for coagula formation. Participation of Ca in coagulum formation is also clearly demonstrated by the reduction of total Ca in the supernatants compared with the Ca content in wheys. However, even at the highest Ca concentration, the amount of the insolubilized material was rather low. About 40 to 60 min were necessary for coagulation. After prolonged hydrolysis, higher NPN in the supernatant and lower NPN in precipitate were detected.

The freeze-dried precipitates (about 1.0 to 1.2 g/100 ml whey) obtained with the highest Ca concentrations (>2000 ppm) contain approximately 24 to 30% protein (N × 6.38), 20 to 30% Ca, and 12 to 14% P. Electrophoresis and HPLC were used to determine the composition of the precipitates obtained (Table 4) from wheys with and without added Ca ions.

The PAGE profiles are shown in Figure 2. Original acid whey (pattern a) exhibited its typical (3, 6) protein components: the slower moving band represented Ig, followed by BSA, α-lactalbumin, and β-lactoglobulin B and A. The precipitate formed in acid whey containing 1714 ppm Ca^{++} (pattern b), in acid whey containing 2514 ppm Ca^{++} (pattern c), and in sweet whey containing 2877 ppm Ca^{++} (pattern d) resolved all three into two equally spaced bands. Identical banding patterns (not shown) were also given by the other precipitates listed in Table 4 (samples d, a2, a3). The slow and the fast-moving bands appeared to correspond in mobility to BSA and α-la, respectively, as compared with original acid whey (pattern a). In reality, these bands represent the products of binding and rearrangement of low molecular weight material produced by the

TABLE 3. Second series: composition of sweet and acid whey samples.

Whey	pH	Titratable acidity	Total protein	NPN	Total Ca^{++}	Ionic Ca
		(°SH)[1]	(%)		(ppm)	
Sweet	6.43	3.1	.60	.25	477	120
Acid	4.64	. . .	.50	.18	1714	1710

[1] °SH = Soxhlet Henkel degrees.

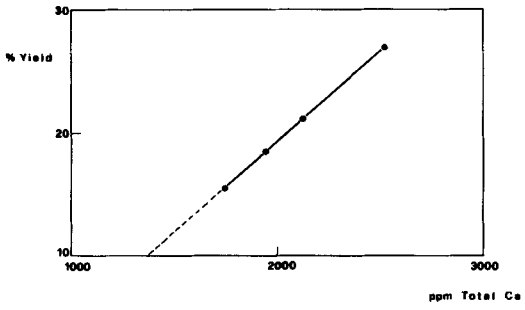

Figure 1. Relationship between total Ca content and alcalase-induced precipitate formation in terms of protein recovery (% yield) in acid whey samples.

enzyme action on protein, as will be shown in the following.

The SDS-PAGE was used to determine whether the components mentioned were built up of polypeptide subunits. Results are given in Figure 3. The main whey proteins (pattern b) were identified, in order of increasing mobility, as BSA, Ig (heavy chain), β-lg, and α-la. The precipitate (Table 4) formed in acid whey containing 1714 ppm Ca^{++} (pattern c), in acid whey containing 2514 ppm Ca^{++} (pattern d), and in sweet whey containing 2877 ppm Ca^{++} (pattern e) all resolved into two very close zones. The other precipitates of Table 4 afforded identical patterns (not shown), although slight differences in staining intensity were observed. No extra components were seen even by loading 500 μg of coagulum on the gel. The molecular weight corresponding to these zones was roughly estimated at 7000 to 10,000 daltons. This suggests that the components of the two bands in which all the precipitates of Table 4 were separated by PAGE are in turn composed of subunits, as they disappeared in the presence of mercaptoethanol.

This separation was consistent with results of HPLC determinations (Figure 4). Chromatogram B of Figure 4 is the profile of the main components of original acid whey. Comparison with retention times of standard proteins (chromatogram C) and with a calibration plot of log molecular weight versus retention time allowed identification of the major peaks. For the original whey, peaks were at 21, 23, 27, and 28 min, corresponding to Ig, BSA, β-lg, and α-la, respectively. The typical

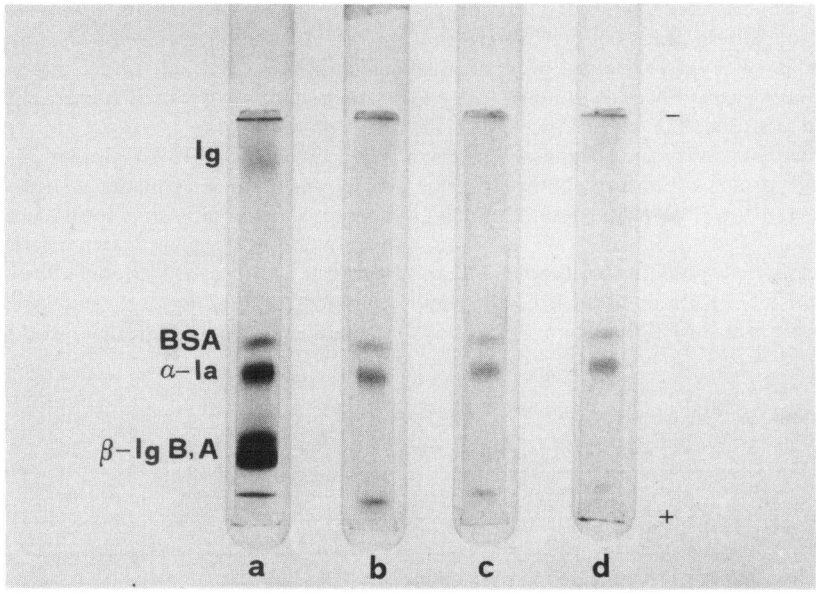

Figure 2. The PAGE patterns of the precipitate induced by alcalase treatment of b, acid whey containing 1714 ppm Ca^{++}; c, acid whey containing 2514 ppm Ca^{++}; d, sweet whey containing 2877 ppm Ca^{++}; a, original acid whey (freeze-dried sample). 200 μg material were loaded on each gel. The fastest moving band is that of the marker dye. BSA = Bovine serum albumin, α-la = α-lactalbumin, β-lg = β-lactoglobulin.

TABLE 4. Second series: precipitate formation in terms of protein recovery from whey samples and total calcium content in wheys and supernatants.

Sample description	Protein recovery	Total calcium	
		In whey[1]	In residual supernatants[2]
	(%)		
From sweet whey			
a, Whey + enzyme	...	477	472
b, Whey + 200 ppm Ca^{++} + enzyme	...	677	676
c, Whey + 500 ppm Ca^{++} + enzyme	...	977	795
d, Whey + 1200 ppm Ca^{++} + enzyme	14.6	1677	1056
e, Whey + 2400 ppm Ca^{++} + enzyme	20.0	2877	1761
From acid whey			
a1, Whey + enzyme	15.4	1714	480
a2, Whey + 200 ppm Ca^{++} + enzyme	18.5	1914	532
a3, Whey + 400 ppm Ca^{++} + enzyme	21.0	2114	594
a4, Whey + 800 ppm Ca^{++} + enzyme	28.0	2514	910

[1] Calculated as sum of initial plus added.

[2] Direct determination.

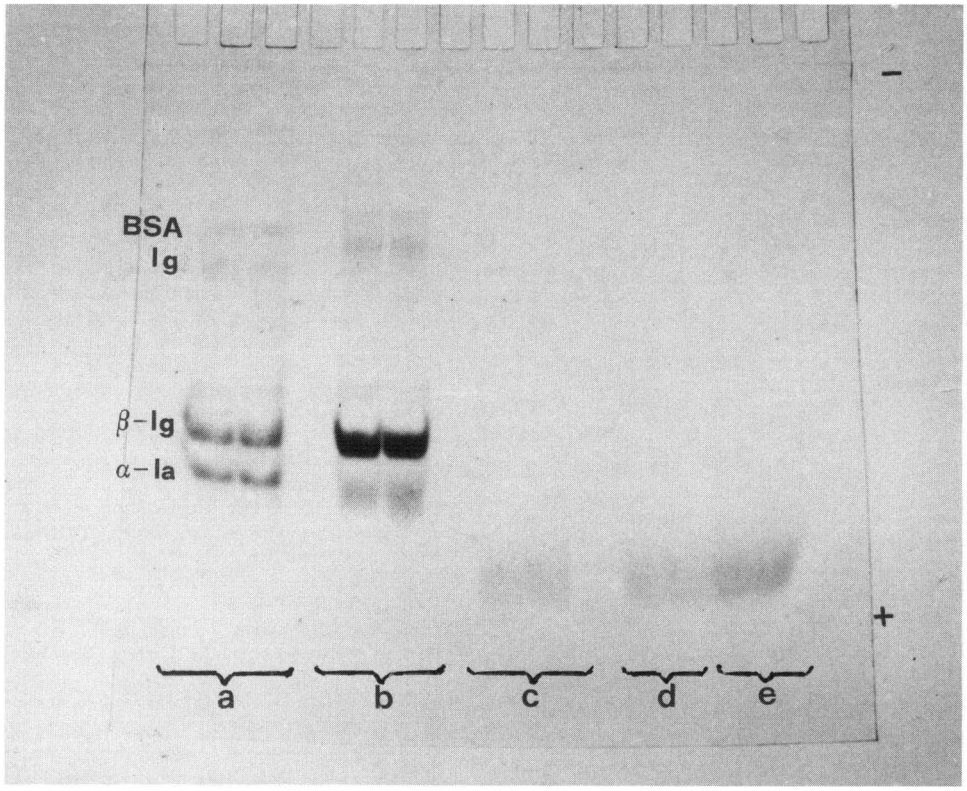

Figure 3. The SDS-PAGE patterns of the precipitate induced by alcalase treatment of c, acid whey containing 1714 ppm Ca^{++}; d, acid whey containing 2514 ppm Ca^{++}; e, sweet whey containing 2877 ppm Ca^{++}. a, standard proteins from top to bottom: bovine serum albumin (BSA, 66,000); IgG, heavy chain ($\sim$ 55,000); β-lactoglobulin (β-lg, 18,400), α-lactalbumin (α-la 14,200); b, original acid whey (freeze-dried sample); material loaded was 50 μg for slot a and 200-300 μg for slots b through e.

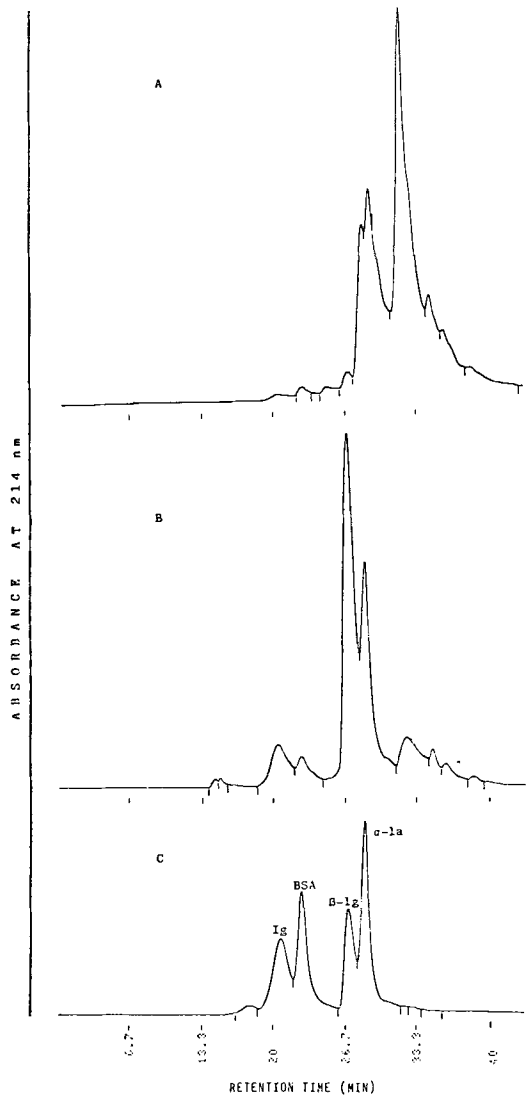

Figure 4. The HPLC elution patterns of A) precipitate induced by alcalase in acid whey containing 1714 ppm Ca^{++}; B) original acid whey (freeze-dried sample); C) standard proteins.

elution pattern of the precipitate collected from the acid whey samples (chromatogram A) was remarkably different, showing, in addition to the peak of the α-la area, at least another main peak shifted toward 34 to 36 min and corresponding to material smaller than α-la.

The two fractions resolved by PAGE (Figure 2, gels b–d) are parts of aggregates assembled with peptides produced from whey proteins by alcalase cleavage; therefore, aggregation was through proteolysis. The aggregate is probably held to a significant extent by very strong noncovalent or covalent (i.e., disulfide) bonds, as it can be dissociated in polypeptide subunits by the presence of detergent (SDS) and mercaptoethanol.

The effect of Ca ions on aggregate formation is not fully understood. Probably Ca^{++} binding neutralizes the net negative charges of peptide fragments, of small-sized aggregates, or of both, causing isoelectric precipitation. Also, as the Ca ions interact with phosphates at alkaline pH (1), the resulting Ca-phosphate complex may change the ion equilibria and partition of salts, causing aggregation and subsequent coprecipitation of peptide material.

A study is underway to assess which protein (or proteins) of the whey is the specific substrate of the alcalase and to verify whether this proteinase preparation mixed with rennet can be advantageously employed in cheese making.

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