Monitoring the Degradation of Commercial Microbial Rennet Preparations[1]

WAYNE LEVADOUX, MARK JOHNSON, and GÉRALD ANDRÉ
National Research Council Canada
Biotechnology Research Institute
6100 Royalmount Avenue
Montréal, Québec, Canada H4P 2R2

ABSTRACT

Fractionation on a DEAE-Sephadex A-50 column was used to study the degradation of microbial rennets derived from *Mucor miehei*. A comparison was made between the crude culture filtrate, derived from the growth of the microorganism in submerged culture condition on wheat bran medium, and commercial preparation Marzyme and Rennilase. The elution protein profiles lacked some of the peaks associated with the crude culture filtrate however, the milk-coagulating activity eluted out at approximately the same NaCl molarity (.45 *M*). Each brand name product had a characteristic elution pattern, which did not vary between their thermostable and thermolabile enzyme preparations. With storage at 4°C the elution protein profiles of the thermolabile enzyme preparations changed in a distinctive manner characteristic of the brand name. With the Marzyme series, the milk-coagulating activity eluted out at the same NaCl molarity, whereas other established peaks increased in area. The milk-coagulating activity, from Rennilase, eluted from the column at a lower NaCl concentration (.35 *M*). The changes were evidence of enzyme degradation, which was manifested by a general decrease in specific enzyme activity. By using column chromatography it was possible to monitor the stages of degradation with time.

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INTRODUCTION

The use of microbial rennets as a substitute for calf rennet is now well-established industrially (5). As of 1974, 60% of the cheese made in the United States was with these substitutes (16). The longevity and thermotolerance of the microbial enzymes, however, cause problems, especially with long-term cheeses such as Cheddar. After the initial operation of curding, a large proportion of the microbial rennet survives the scalding phase and continue to hydrolyze protein, resulting in greater cheese losses and the production of bitter flavors (3, 15). The continuing proteolytic activity causes even greater problems to the whey utilization industry: indeed, the enzymes still coagulate milk proteins when whey is added to milk or cause proteolysis of proteinaceous materials added to whey.

In order to address these concerns, the enzyme-producing industry have developed thermolabile and extra-thermolabile milk-coagulating enzymes, the latter being more thermolabile then calf rennet.

In this study four such thermolabile enzyme preparations were separately fractionated on a DEAE-Sephadex A-50 column and compared with a crude culture filtrate. These commercial preparations were stored at 4°C and sampled periodically to observe variations in their respective fractionation pattern with time. This change in pattern was then correlated to a general decline of specific activity in the commercial preparation.

MATERIALS AND METHODS

Materials

Crude milk-coagulating protease was obtained from the growth of *Mucor miehei* strain Cooney et Emerson CBS 730.65 on bran me-

dium. Commercial microbial rennets Marzyme and Rennilase were gifts from Marschall Products (Miles Laboratories Inc., Madison, WI), and Novo Industry A/S Enzymes Division (Bioindustrial Group, Lachine, P.Q., Can.), respectively. Calf rennet (EC 3.4.23.4), α-amylase derived from *Aspergillus oryzae* (No. A-0273) (EC 3.2.1.1), bovine serum albumin, carboxymethyl cellulose (CMC), low viscosity, and triolein (glyceroltrioleate), 99% pure, were purchased from Sigma Chemical Co. (Sigma Chemical Co., St. Louis, MO). The DEAE-Sephadex A-50 (Lot No. KL 38694, 3.5 meq exchange capacity/g) was from Pharmacia Fine Chemicals (Baie D'Urfé, P.Q., Can.). The Phasebas amylase test kit was supplied by Pharmacia Diagnostics. Instant NDM was Carnation's commercial product. All other chemicals were reagent grade and solutions were made with distilled water.

Media

The bran medium consisted of 60 g of wheat bran and 100 ml of a .1% (wt/vol) solution of ammonium sulfate in a 500-ml Erlenmeyer flask. Flask contents were autoclaved at 121°C for 15 min.

Mucor miehei (CBS 370.65) was propagated and maintained on yeast-malt extract (YM) (Difco Corp., Detroit, MI) agar at 37 and 4°C, respectively (6).

Production of Crude Enzyme

Roux bottles containing YM agar were inoculated with *M. miehei* spores and incubated for 5 to 7 d at 37°C. Using a .01% (wt/vol) Tween 85 solution, a spore suspension was made from each bottle and filtered through sterile glass wool. Ten milliliters of the resulting filtrate were then used to inoculate the bran media contained in 500-ml Erlenmeyer flasks. The inoculated bran medium was subsequently incubated at 37°C for 5 d. The proteolytic enzyme resulting from the growth of the mold on the medium was then extracted by adding 150 ml of sterile distilled water to the contents of each flask. The contents of the flasks were agitated at 300 rpm in a New Brunswick G24 shaker (New Brunswick Scientific Co., Inc., Edison, NJ) for 10 min at room temperature. The flask contents were filtered through Whatman No. 1 filter paper and the filtrate centrifuged at 10,000 × *g* for 15 min at 4°C. The supernate was then distributed among several shallow dishes and freeze dried. The freeze-dried supernate, designated crude enzyme, was then stored at −70°C.

Fractionation of Enzyme Solution

Crude and commercial enzyme samples were dialyzed against 17 vol of .05 *M* sodium acetate buffer (pH 5.6) at 20°C using an Amicon filtration unit (Amicon Canada Ltd., Oakville, Ont., Can.) equipped with a YM 10 membrane. The retentates (3.0 ml) were then fractionated on a DEAE Sephadex A-50 column with an average bed volume of 113 ml using an average flow rate of .7 ml/min against an increasing stepwise NaCl gradient (0 to .7 *M*) at 20°C. The salt gradient was measured with a conductivity meter (model C 50 D) supplied by Canlab (6800 Trans-Canada Road, Pointe Claire, P.Q., Can.). Four-milliliter fractions were collected with an LKB 2211 Superrac fraction collector equipped with a LKB 2158 Uvicord SD spectrophotometer (280 nm) and LKB 2210 recorder (Pharmacia Canada Inc., 500 Morgan Blvd., Baie d'Urfé, P.Q., Can.). Protein levels in individual fractions were monitored at 280 nm in a Bausch and Lomb Spectronic 2000 spectrophotometer.

Protein Determination

The Folin reaction (10) was used to determine protein concentration in samples of eluates obtained after fractionation of the commercial products and of the crude enzyme. Bovine serum albumin was used as the standard reference protein.

Milk-Coagulating Activity Determinations

The coagulating activity was determined at 37°C as described by Conelius (4). The substrate consisted of a suspension of 10% (wt/vol) NDM solids solubilized in .01 *M* CaCl solution with a final pH of approximately 6.1. One Soxhlet unit (SU) was defined as the amount of enzyme activity that could clot 1 ml of the milk solution in 40 min at 37°C.

Amylase Activity

To 4 ml of distilled water was added one tablet from the phadebas amylase test kit. The

contents were agitated in a Vortex mixer for 5 s and incubated for 5 min at 37°C in a water bath. To this suspension was added .2 ml of a diluted enzyme sample, the mixture was agitated for 5 s, and then returned to the water bath for 5 min. The reaction was stopped by the addition of 1 ml of .5 N NaOH followed by agitation. Four milliliters of the sample were centrifuged at 1500 × *g* for 5 min, and the resulting supernate was measured at 620 nm for color development. α-Amylase derived from *A. oryzae* was used as the standard reference for activity.

Carboxymethylcellulase Activity

One milliliter of a diluted enzyme solution was added to 1 ml of a 4% (wt/vol) CMC solution in .2 *M* acetate buffer at pH 5.0. The contents were agitated and then incubated at 50°C for 20 min. The reaction was stopped by placing the test tubes on ice and then adding 3 ml of a dinitrosalicylic acid (DNS) solution, described previously (11). Color was developed by heating the solution in screw cap tubes to 95°C for 15 min and then cooling down to 4°C. Samples were then diluted to 15 ml with distilled water and absorbance was measured at 640 nm. Dextrose was used as the standard reduced sugar reference for the DNS method. One unit of activity was defined as the equivalent production of 1 μg of reduced sugar/min per ml of enzyme solution added.

Lipase Activity

To .5 ml of triolein was added .5 ml of the diluted enzyme sample suspended in .05 *M* acetate buffer pH 5.6. The mixture was incubated at room temperature with wrist action agitation on a Burrell Wrist Action agitator (Burrell Corp., Pittsburgh, PA) for 24 to 48 h. A 5-μl homogeneous sample was then added to 200 μl of acetone and analyzed by HPLC procedures as described previously (F. Ergan, 1988, unpublished). Lipase activity was based on the percent triolein remaining after 24 h incubation.

Thermostability Assay

Each commercial enzyme product was diluted in equal amounts of .05 *M* acetate buffer to a final pH value of 6.5. The calf rennet was first dialyzed against 90 ml of the same acetate buffer, using an Amicon filtration unit equipped with a YM 10 membrane, and adjusted to a final pH of 6.7 with acetic acid. The diluted enzyme preparations were then incubated at 70°C for 0, 5, 10, and 15 min and then cooled to 4°C on ice. A sample from each time interval was taken and the remaining milk-coagulating activity was determined.

RESULTS

Enzyme Survey

Comparison of a crude *M. miehei* culture filtrate and the commercial enzyme preparations revealed differences in enzyme composition. The crude filtrate contained milk-coagulating enzyme (3934 SU/ml), amylase (990 U/ml), CMC (15,404 U/ml), and lipase (11.4% triolein remaining after 24 h) activities. The commercial enzymes processed only milk-coagulating activity.

A comparison of the elution profiles on DEAE Sephadex A 50 obtained with the crude and commercial enzymes revealed both differences and similarities (Figures 1 to 3). Figure 1 illustrates the standard profile of the crude culture filtrate: seven major protein peaks can be seen. The elution position of all the enzymes assayed except for lipase were located: CMC activity at .22 *M* NaCl, amylase I at .35 *M*, amylase II at .40 *M*, and the milk-coagulating activity at .45 *M* NaCl. Initially (d 0), with the Marzyme and Rennilase enzymes, similarities

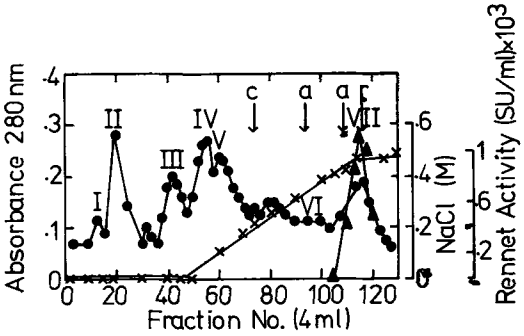

Figure 1. Fractionation of crude *Mucor miehei* culture filtrate on DEAE Sephadex A-50 and location of carboxymethylcellulase (c), amylase (a), and milk-coagulating (rennet) (r) activity. Absorbance (•), NaCl gradient (x), and rennet activity (▲). SU = Soxhlet unit.

in the profiles were noticed (Figures 2 and 3). Most contained the corresponding peaks I, II and VII of the crude enzyme profile with peak VII always containing the milk coagulating activity eluting at approximately .45 *M* NaCl. Differences between the commercial enzymes were also observed; Figure 3 shows that peaks III, IV, and V were less pronounced with the Rennilase preparations.

Effect of Storage Time

As storage time continued, the protein profiles of each enzyme preparation changed.

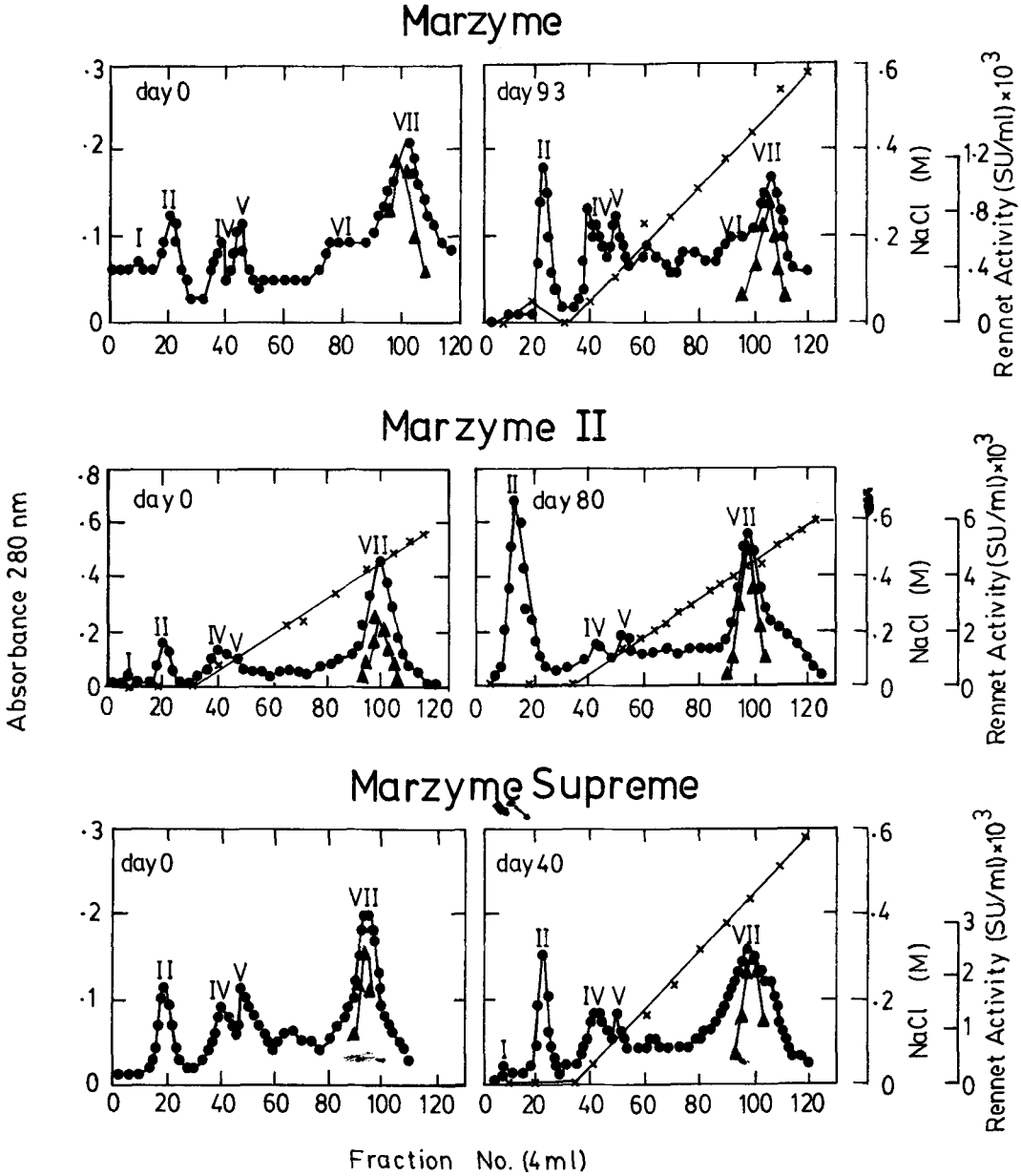

Figure 2. Fractionation of the Marzyme preparations. Absorbance (•), NaCl gradient (x), and rennet activity (▲).

In the Marzyme series (Figure 2), the milk-coagulating peak (VII) still eluted out at .45 *M* NaCl while peak II increased in area (i.e., Marzyme II). With the Rennilase preparations (Figure 3), peak VII of d 0 changed into two peaks with the milk-coagulating activity eluting out at .30 to .35 *M* NaCl instead at .45 *M* NaCl. These changes in elution profiles are probably related to modifications in the specific activity of these enzymes (Table 1). Marzyme's unfractionated and fractioned specific activities did not vary within the 93 d of storage, however; such was not the case for the thermolabile enzyme preparations. Specific activities of Marzyme Supreme were not determined at all stages of storage; however, studies with Marzyme II revealed respective losses and gains with the unfractionated and fractionated samples. This same phenomenon was repeated with Rennilase L Type TL and Rennilase L Type XTL, the unfractionated specific activity declined whereas that of the fractionated increased.

Thermostability

Variations (± 5%) in thermostability were observed among the commercial microbial rennets and when compared to calf rennet following exposure to 70°C (Table 2). The most stable was the Marzyme preparation, in which 87% of the original activity remained after 15 min exposure at 70°C. The most labile preparations were Marzyme Supreme and Rennilase L Type XTL, which are claimed by the suppliers to be similar to calf rennet with regards to temperature stability. Each showed only .7% activity remaining after 15 min exposure, whereas calf rennet (Sigma) had 9 to 15% activity remaining after 10 and 15 min,

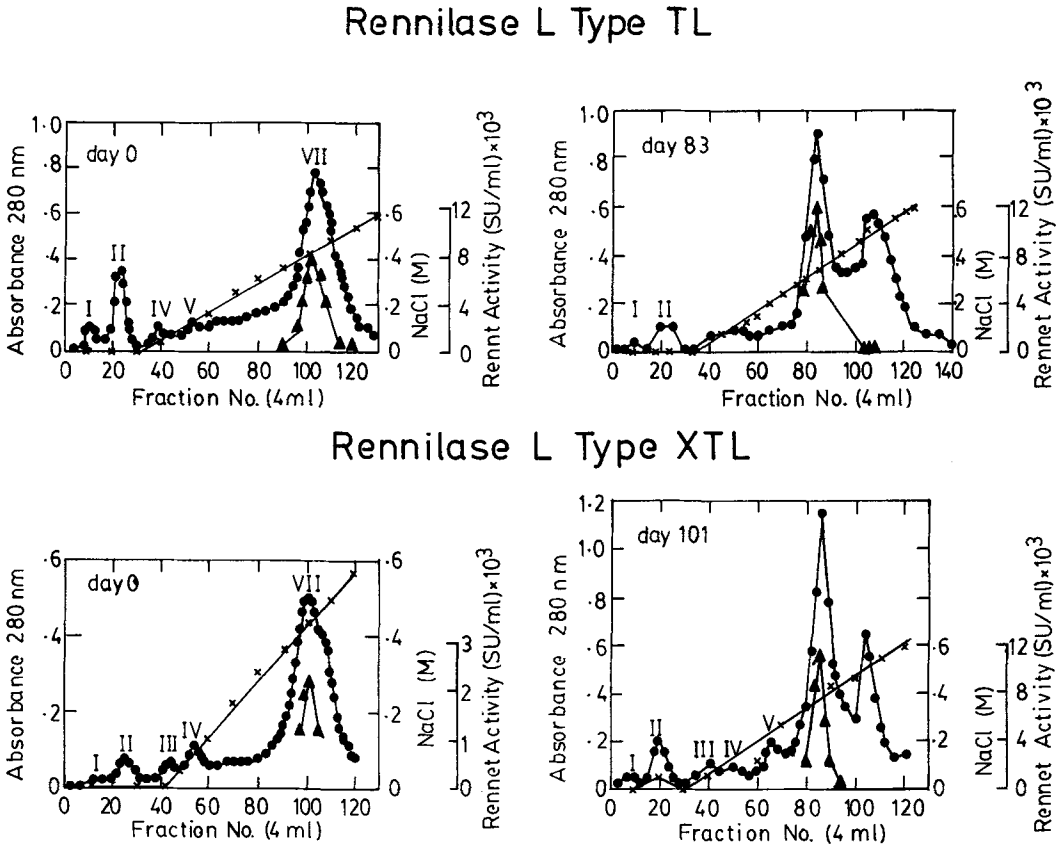

Figure 3. Fractionation of Rennilase preparations. Absorbance (•), NaCl gradient (*), and rennet activity (▲).

TABLE 1. The effect of storage time at 4°C on the specific activity of commercial microbial rennets.[1]

Enzyme source	Storage time	Specific activity[2]			
		Unfractionated		Fraction[3]	
		Initial 1	Final 2	Initial 1	Final 2
	(d)	(SU/μg protein)[4]			
Marzyme	93	3.3	3.4	10.5	10.9
Marzyme II	80	5.0	3.7	6.6	10.8
Marzyme Supreme	. . .	4 1	. . .	2.5	. . .
Rennilase L Type TL	83	5.3	3.1	10.5	14.7
Rennilase L Type XTL	101	5.4	3.5	4.7	10.8

[1] Enzymes were all maintained at 4°C.

[2] Milk-coagulating activity was determined from dilutions made in .05 *M* acetate buffer (pH 5.6).

[3] Milk-coagulating activity in fractions containing on average .4 *M* NaCl in .05 *M* acetate buffer (pH 5.6) following fractionation on DEAE Sephadex A-50.

[4] SU = Soxhlet units.

TABLE 2. A comparison of thermostability between calf and commercial microbial rennets.

Enzyme sample	Scalding time[1]	Activity remaining[2]
	(min)	(%)
Marzyme	0	100
	5	104
	10	96
	15	87
Marzyme II	0	100
	5	34
	10	23
	15	10
Marzyme Supreme	0	100
	5	20
	10	3
	15	.7
Rennilase L Type TL	0	100
	5	45
	10	26
	15	22
Rennilase L Type XTL	0	100
	5	22
	10	2
	15	.7
Calf Rennet	0	100
	1	30
	5	8
	10	9
	15	15

[1] Enzyme solutions adjusted to pH 6.5 or 6.7 in .05 *M* acetate buffer were subjected to 70°C for 0 to 15 min.

[2] The percent milk-coagulating activity remaining in each solution was calculated from the activity at time zero.

respectively. Marzyme II and Rennilase L Type TL showed results similar to calf rennet with 10 and 22% activity remaining, respectively. Except for the unmodified enzyme sample, Marzyme, all products exhibited precipitation after exposure to 70°C.

DISCUSSION

In an initial step, a comparison was made between the crude *M. miehei* culture filtrate and the commercial rennets (Marzyme and Rennilase) to determine whether or not some enzymatic activities had been removed during the downstream processing of the commercial enzymes. Because all the commercial enzymes were derived from *Mucor miehei* [(12), Miles Product Service, and Novo Industri.], the initial milk-coagulating activity eluted out at approximately the same NaCl molarity (.45 *M*) as that found with the crude filtrate prepared by us. The main difference between the commercial enzymes and the crude filtrate was the absence of specific enzymic activities (amylase, CMC, and lipase) in the commercial preparations. It is not known whether the undesirable enzymes were removed or destroyed. However, comparison of the elution protein profiles between the commercial products suggested processing variations. Sternberg (15) described a procedure using reversible protein precipitation with polyacrylic acids for the purification of a milk-coagulating enzyme. Other classical procedures for precipitating proteins exist, such as the use of ammonium sulfate or acetone, but the objective of these procedures is to remove the undesirable in favor of the target enzyme.

Another tactic of removing undesirable enzymes is to destroy them during processing. The methods are many, but in all cases, the objective is to treat the fermentation filtrate in such a manner as to favor the survival of the desired enzyme. For example, Kokusho et al. (9) described a procedure for inactivating cellulase in a culture filtrate by heat treatment in the presence of 5 to 77% NaCl. Schleich (13) inactivated esterases by maintaining the culture filtrate at pH 2.0 to 3.5 at 20°C until no activity remained. Whatever the procedure used by the commercial enzyme producers, no residual enzyme activity other than milk coagulation was found. This is contrary to earlier findings by Shehata et al. (14) where α-amylase was associated with the Rennilase product.

Concentrating on the milk-coagulating factor, thermotolerance variations were observed among the brand name products, although there was no obvious difference in the protein elution profiles for these enzymes at d 0. Marzyme, the thermostable microbial rennet, retained 87% of its original activity after 15-min exposure at 70°C. The industrially treated enzyme preparations Marzyme II and Rennilase L Type TL, however, retained only 10 to 22% activity, respectively, a result similar to that obtained with calf rennet. Marzyme Supreme and Rennilase L Type XTL, both designated as more thermolabile than calf rennet, had less than 1% activity remaining (Table 2). These thermotolerance findings were contrary to the printed material supplied by the companies (Miles Product Service and Novo Industri). These differences may be attributed to variations in assay conditions; indeed, the enzyme supplier(s) used reconstituted whey and the pH was adjusted with lactic acid as opposed to suspending the enzymes in buffer. The increased salt concentrations in whey and the smaller enzyme concentrations presumably used in their experiments may have lead to the increased sensitivity of the microbial rennets to the temperatures. The thermal variation between thermostable and thermolabile enzymes was attributed to chemical treatments aimed at oxidizing sensitive areas of the protein. Again, the methods are many but the objective is the oxidation of methionine and histidine residues in the protein with chemicals such as hydrogen peroxide, dyes, and hypochlorites (2, 4, 8). Using NMR analysis, Branner et al. (1) found the most pronounced effect of chemical oxidation is the formation of methionine sulfoxides. Exposing the microbial rennets to oxidizing agents for longer periods results in greater thermal instability.

Commercial microbial rennets are said to deteriorate naturally with time depending on storage temperature (Miles Product Services and Novo Industri). Enzyme deterioration is usually manifested by a decrease in enzyme activity. Experiments were performed in order to evaluate enzyme deterioration differently and to examine more closely the effect of this natural enzyme deterioration on the extremely

thermolabile enzymes. As expected, the overall specific activity of the enzymes declined with time (Table 1). Examination of the enzyme preparations by protein profile elutions on DEAE-Sephadex A-50 revealed alterations in the patterns of the peaks for the milk coagulating enzymes with storage; in addition, the specific activity of the milk-coagulating enzymes associated with these peaks increased during storage. With the Marzyme series, the milk-coagulating enzymes (peak VII), after prolonged storage at 4°C, eluted out at the same NaCl molarity while other established peaks (i.e., peak II) tended to increase in size (Marzyme II and Marzyme Supreme). These changes are assumed to result from degradation of the enzymes. During storage at 4°C, the loss in overall specific activity was accompanied by an increase in the nonspecific protein peak II. With the Rennilase series, the elution pattern of the product as well as the pattern of degradation was different from Marzyme. The Rennilase product had smaller nonspecific protein peaks (III, IV, and V) and, after prolonged storage at 4°C, the microbial rennets eluted out of the DEAE-Sephadex A-50 column at a lower NaCl concentration (.35 *M*) than the nondeteriorated form or the Marzyme product (.45 *M*). This resulted in the presence of an extra peak in the protein elution profiles (Figure 3). Once again, the specific activity of the microbial rennet associated with the active peak had increased during prolonged storage at 4°C while that of the overall enzyme preparation decreased.

Our study shows conclusively that Marzyme preparations can be distinguished from the Rennilase by fractionation on DEAE-Sephadex A-50. This procedure, however, cannot distinguish between thermostable and thermolabile enzymes within the same brand name (Marzyme or Rennilase). The degradation (or deterioration) of these enzymes, especially the thermolabile ones, can be easily monitored. The Marzyme and Rennilase enzymes, when stored at 4°C for prolonged times, deteriorate in a different fashion. This is probably due to differences in the processing of these enzymes and one the procedures used to increase thermolability. Our lack of information on these matters makes it difficult to explain why the Marzyme thermolabile rennets deteriorate differently from the Rennilase rennets.

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REFERENCES

1 Branner, S., P. Eigtved, M. Christensen, and H. Thogersen. 1984. Amino acid and NMR analysis of oxidized *Mucor miehei* rennet. Enzyme Eng. 7:434.
2 Branner-Jorgensen, S., P. Schneider, and P. Eigtved. 1982. Thermal destabilization of microbial rennet. US Pat. 4,357,357.
3 Cheesman, G. C. 1980. Rennet and cheesemaking. Page 195 *in* Enzymes and food processing. G. G. Birch, N. Blakebrough, and K. J. Parker, ed. Appl. Sci. Publ. Ltd., London, Engl.
4 Cornelius, D. A. 1982. Process for decreasing the thermal stability of microbial rennet. US Pat. 4,348,482.
5 Cruegar, W., and A. Cruegar. 1984. Enzymes. Biotechnology: a textboox of industrial microbiology. T. D. Brock, ed. Sci. Tech., Inc., Madison, WI.
6 Difco Manual. 1984. Page 1132 *in* Dehydrated culture media and reagents for microbiology. 10th ed. Difco Labs., Detroit, MI.
7 Reference deleted in proof.
8 Etoh, Y., H. Shoun, K. Arima, and T. Beppu. 1982. Photooxidation of a hystidyl residue of milk-clotting acid protease, mucor rennin. J. Biochem. 91:747.
9 Kokusho, Y., S. Higashi, H. Machida, and S. Iwasaki. 1976. Process for improving the quality of microbial rennet. US Pat. 3,950,221.
10 Lowry, O., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265.
11 Miller, G. L., R. Blum, W. E. Glennon, and A. L. Burton. 1960. Measurement of carboxymethylcellulase activity. Anal. Biochem. 2:127.
12 Nelson, J. H. 1974. Symposium: application of enzyme technology to dairy manufacturing. J. Dairy Sci. 58:1739.
13 Schleich, H. 1981. Removing esterase from microbial rennin. US Pat. 3,616,233.
14 Shehata, A. E., A. A. Ismail, A. Hegazi, and A. M. Hamdy. 1978. Fractionation of commercial rennet enzymes on Sephadex G-100. Milschwissenschaft 33:693.
15 Sternberg, M. 1976. Purification of industrial enzymes with polyacrylic acids. Process Biochem. September: 11.
16 Sternberg, M. 1976. Microbial rennets. Adv. Appl. Microbiol. 20:135.