0273-2289/93/37(3)—0283$03.40

Purification and Characterization of a Milk Clotting Protease from *Mucor bacilliformis*

LILIANA B. ARECES,[1]
MIRTHA BISCOGLIO DE JIMÉNEZ BONINO,[1]
MARINA A. A. PARRY,[1] ELDA R. FRAILE,[2]
HÉCTOR M. FERNÁNDEZ,[2] AND OSVALDO CASCONE*,[2]

[1]*Instituto de Quimica y Fisicoquimica Biológicas (UBA-CONICET);*
and [2]*Cátedra de Microbiologia Industrial y Biotecnologia, Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires. Junin 956 (1113) Buenos Aires, Argentina*

Received June 17, 1992; Accepted September 22, 1992

ABSTRACT

An acid protease having milk clotting activity has been isolated from *Mucor bacilliformis* cultures. The enzyme was basically purified by ionic exchange chromatography. An average yield of 29 mg purified product was obtained from 100 mL crude extract. As purity criteria, SDS-PAGE, reverse-phase HPLC, and N-terminal analysis were performed. The protease is a protein composed of a single polypeptide chain with glycine at the N-terminus. The mol wt is approx 32,000, and its amino acid composition is very similar to those of other fungal proteases. As expected, its clotting activity was drastically inhibited by pepstatin A action. On the other hand, its instability against heat treatment and its clotting/proteolytic activity ratio indicate that it may be considered as a potential substitute for bovine chymosin.

Index Entries: *Mucor bacilliformis* protease; milk clotting enzyme; acid protease; fungal protease; aspartyl protease.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

For a long time, the most widely used source of milk clotting enzymes for cheese manufacture was the stomachs of young animals. However, their decreasing supply and the increase in world cheese production and consumption have made utilization of "rennet" substitutes necessary.

The first stage in cheese manufacture involves the hydrolysis of the peptidic bond between Phe 105 and Met 106 (*1*) or Ser 104 and Phe 105 (*2*) of the k-casein leading to milk coagulation in the presence of calcium. The last step—in which proteolytic activity predominates—deals with the aging of cheese. Bovine chymosin fully qualifies for this purpose, since it has a high clotting activity, but ensures that only a limited number of peptidic fragments is released during the maturation process. This is the main problem of many substitutes studied: Their excessive proteolytic activity results in the appearance of bitter peptides that impair the cheese flavor. Among alternatives investigated, microbial substitutes—such as those from *Mucor miehei* and *Mucor pusillus*—are of great importance. However, both strains are thermofilic, and the corresponding proteases are more stable to heat treatment than bovine chymosin. In a previous paper, Fraile et al. (*3*) reported the presence—in a crude extract from a mesofilic strain of *Mucor bacilliformis*—of milk clotting activity having low heat stability.

This article reports the isolation and purification—from a local strain of the above mold—of an acid protease suitable for cheese manufacture. In order to characterize it, its molecular weight, the effect of an aspartyl-protease-specific inhibitor on its clotting activity, the enzyme stability against heat treatment, optimum pH, and K_m value were determined. In addition, the amino acid composition and N-terminal residue determination allowed the establishment of some comparisons with other milk clotting proteases—including those from fungal origin currently used in cheese manufacture.

MATERIALS AND METHODS

Mucor Strain

The *Mucor bacilliformis* strain was obtained from a collection of Biochemistry and Pharmacy School (Buenos Aires, Argentina). Bovine serum albumin (Fraction V), DEAE-cellulose medium mesh, Type I hemoglobin, pepstatin A, *p*-chloromercuriphenyl sulfonic acid (p-CMPS), phenylmethylsulfonyl fluoride (PMSF), and dansyl chloride were obtained from Sigma Chem. Co.USA. Wheat bran was from Morixe, Argentina; Hammarsten-grade casein was from Merck, Darmstadt, Germany; Peake grade A skim milk powder was from Galloway West, USA. All other reagents were AR grade.

Mold Culture

Wheat grains were used as a sporulation medium as previously described (*3*). Cultures were grown in 300-mL Erlenmeyers on 10 g wheat bran moistened with 200 m*M* HCl (*4*) up to a water content of 120% on a dry basis, and autoclaved for 30 min at 121°C. Each Erlenmeyer was inoculated with 1×10^5 spores/g of wheat bran, and cultures were developed at 24°C.

Extraction of the Enzyme

Cultures were extracted with 100 mL of distilled water by shaking at 220 rpm for 1 h, and were then centrifuged at 2500*g* for 20 min at 5°C.

Milk Clotting Activity Determination

It was performed by the procedure of Arima et al. (*5*). The unit of milk clotting activity (CAU) is defined as the enzyme amount capable of clotting 1 mL of substrate in 40 min at 35°C. A 10% solution of skim milk powder in 10 m*M* calcium chloride was used as substrate.

Proteolytic Activity Determination

The method described in the Food Chemical Codex (*6*) was used. The unit of proteolytic activity (PAU) is defined as the enzyme amount capable of releasing 1 μg of tyrosine/min.

SDS Polyacrylamide Gel Electrophoresis

It was performed as described by Laemmli (*7*). Stacking gels contained 5%, and separating gels 10% acrylamide. Gel staining with Coomassie Brilliant Blue R-250 was done by the method of Weber and Osborn (*8*), except for the destaining being achieved by simply shaking the gels in the destaining solvent.

Clotting Activity Detection on the Gel Bands

The procedure of Shovers and Fossum (*9*) was used.

Protein Concentration

It was measured according to Lowry et al. (*10*).

DEAE Cellulose Chromatography and Rechromatography

Sixty milliliters of extract, exhaustively dialyzed against 50 m*M* sodium phosphate buffer, pH 5.8, containing 70 m*M* of sodium chloride

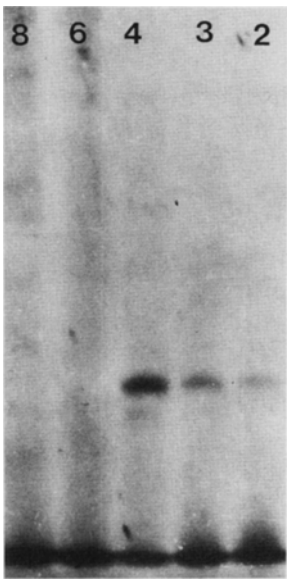

Fig. 1. SDS-PAGE of crude extract from *Mucor bacilliformis* culture. Numbers represent days of culture evolution.

were chromatographed on a DEAE-cellulose column of 7×2.7 cm equilibrated with the same buffer: The first 240 mL were eluted with the same buffer, and then the sodium chloride concentration in the buffer was increased stepwise or by linear gradient up to 170 m*M*. Flow rate was 1 mL/min, and 4-mL fractions were collected. The eluent was monitored at 220 nm, and clotting activity was determined in all the fractions. Fraction B (Fig. 2) was dialyzed against 50 m*M* sodium phosphate buffer containing 70 m*M* sodium chloride rechromatographed under the same conditions, but using a 70–170 m*M* sodium chloride gradient (Fig. 3).

N-Terminal Residue Determination

The method of Gray (*11*) was used.

Reverse-Phase High-Performance Liquid Chromatography

A Waters chromatograph and an Aquapore BU 300 (220×2.1 mm) column was used. Samples were injected and eluted with a 5–70% isopropanol gradient in 0.1% trifluoroacetic acid. Volume gradient was 30 mL, flow rate 0.3 mL/min, and the effluent was monitored at 220 nm.

Amino Acid Analyses

Samples were hydrolyzed at 110°C for 20 h in vacuum-sealed tubes with constant boiling HCl containing phenol (1 mg/mL). The hydrolysates were analyzed in a Beckman 119 CL autoanalyzer.

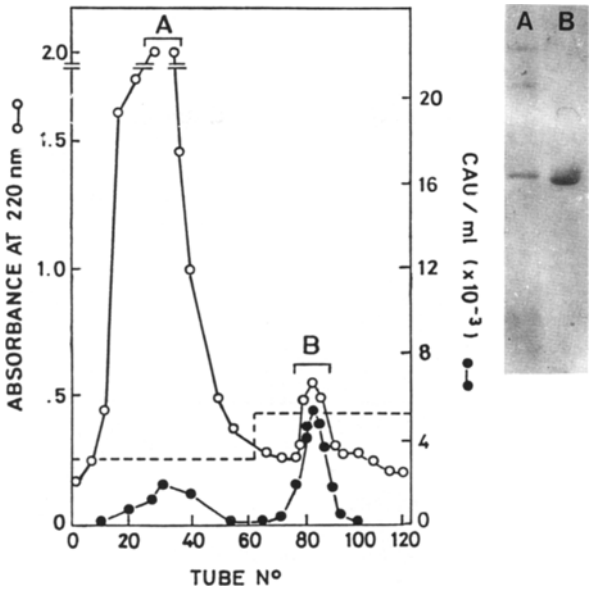

Fig. 2. Crude extract chromatography on a DEAE-cellulose column (7×2.7 cm) equilibrated with 50 m*M* phosphate buffer, pH 5.8, and eluted with a discontinuous gradient: the same buffer containing 70 and 170 m*M* sodium chloride during the first and second step, respectively. Flow rate was 1 mL/min. Four-milliliter fractions were collected. Absorbance at 220 nm and clotting activity of the fractions were measured. The insert shows the SDS-PAGE pattern of fractions A and B.

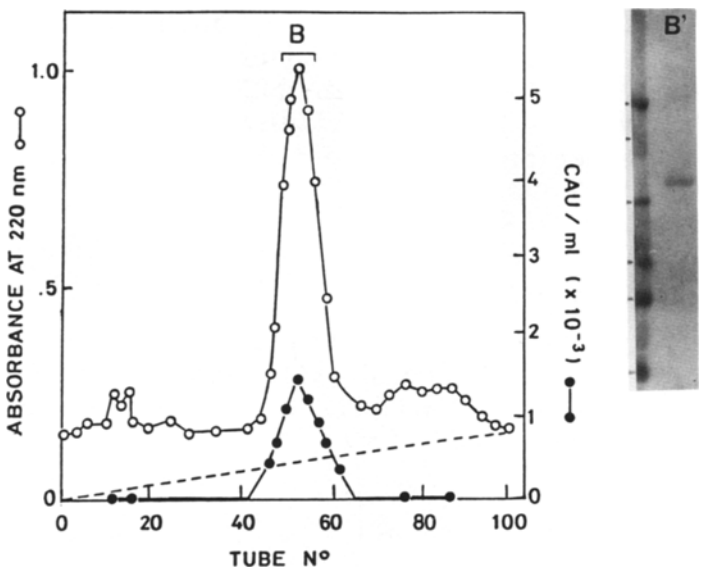

Fig. 3. Rechromatography of fraction B (Fig. 2) on a DEAE-cellulose column (7×2.7 cm) equilibrated with 50 m*M* phosphate buffer, pH 5.8, and eluted with a gradient from 90 to 170 m*M* sodium chloride in the same buffer. Flow rate was 1 mL/min. Two-milliliter fractions were collected. Absorbance at 220 nm and clotting activity of the fractions were measured. The insert shows the SDS-PAGE pattern of fraction B.

Table 1
Mucor bacilliformis Culture Evolution

Culture, d	Protein, mg/mL	CA, CAU/mL	PA, PAU/mL	CA/PA
2	5.4	5714	15	380.9
3	5.8	6667	14	476.2
4	6.2	6316	23	274.6
5	6.2	450	5	90.0
6	6.1	300	4	75.0

Optimum pH Determination

It was determined by measuring proteolytic activity with both 1% hemoglobin and 0.7% casein as substrates.

Heat-Stability Evaluation

It was performed by incubating 1 mL of an aqueous solution of the purified protease (0.14 mg/mL) at preset temperatures for 30 min. Clotting and proteolytic activity were subsequently measured.

K_m Determination

A casein solution in 50 m*M* Gly-HCl buffer, pH 3.0, was used as substrate (concentrations ranging between 0.02 and 0.8% were used), and proteolytic activity was determined. K_m value was obtained by applying a nonlinear regression program in order to fit the data to a Michaelis-Menten curve.

Influence of Protease Inhibitors on Enzymatic Activities

Clotting activity was assayed in the presence of pepstatin A (concentration ranging between 10 and 10^{-3} μM). Proteolytic activity was measured after a 30-min incubation period at 35°C in the presence of 2.5 m*M* EDTA, 5 m*M* PMSF, or 1 m*M* *p*-CMPS.

RESULTS AND DISCUSSION

Culture Evolution

Table 1 summarizes the main characteristics of the evolution of a *Mucor bacilliformis* culture. Extract protein concentration increases as the culture develops until it reaches values that become stable between the fourth

Table 2
Clotting and Proteolytic Activities of Fractions A and B (Fig. 2)

Fraction	CA, U	SCA[a], U/mg	PA, U	CA/PA
A	1804	530	8	225.5
B	11736	10030	16	733.5

[a]Specific clotting activity.

and sixth day. Milk clotting activity increases until it reaches a maximum on the third day. The proteolytic activity achieved a maximum value on the fourth day and then becomes stable.

Both activities show a significant drop as of the fifth day. Moreover, on the third day, the culture shows a good clotting activity and a clotting/proteolytic activity ratio similar to that of other industrial substitutes.

In order to evaluate the complexity of the system, culture extracts were submitted to SDS-polyacrylamide gel electrophoresis (Fig. 1). In 2-, 3-, and 4-d cultures, there is a major band with a mol wt of 32,000. This mol wt value is characteristic of acid proteases from different sources. The band intensity increases until the fourth day, but then starts dropping. As of the fourth day, the 32,000 mol-wt band is accompanied by others of a higher mol wt. The former disappears as of the sixth day.

When a mixture of extracts from the third and seventh days was incubated at 21°C during 2 h, the milk clotting activity was lost and the 32,000-mol-wt band disappeared. Heating of the seventh-day extract at 100°C during 10 min prevents this effect, thus suggesting the presence of a thermolabil factor able to degrade the protease having clotting activity.

On the other hand, in order to confirm that the 32,000-mol-wt band corresponds to an acid protease, clotting activity was expressed *in situ* after SDS-PAGE of the third-day extract. The Shovers and Fossum method allowed visualization of filamentous strands of clotted milk embedded in the gel on the 32,000 band, thus indicating its clotting activity (not shown). In view of these results, the purification procedure was undertaken by using the third-day extract.

Purification Procedure

Figure 2 shows the DEAE-cellulose chromatography elution pattern of the previously dialyzed crude extract. The insert shows the SDS-PAGE pattern of fractions A and B. Both fractions have clotting activity, but on the basis of their specific activity and the clotting/proteolytic activity ratio (Table 2), fraction B was selected for further purification.

The fraction rechromatography elution pattern is shown in Fig. 3. Reverse-phase HPLC, SDS-PAGE, and N-terminal residue determination were performed as purity criteria of the protein. Only one fraction was

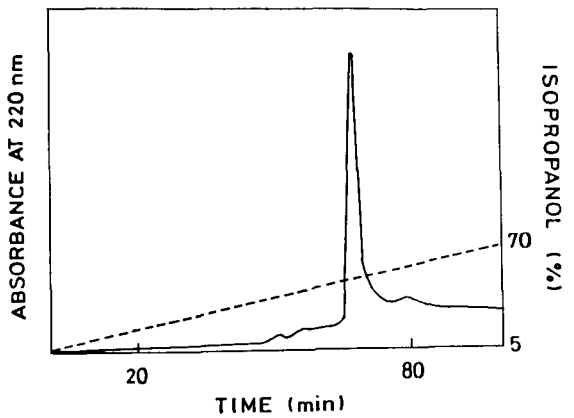

Fig. 4. Elution pattern of fraction B on an Aquapore BU-300 column (220×2.1 mm). The gradient was 5–70% isopropanol in 0.1% trifluoroacetic acid at a flow rate of 0.3 mL/min. The effluent was monitored at 220 nm.

evidenced by both HPLC and SDS-PAGE, and Gly was obtained as the sole N-terminal residue, thus indicating the presence of a homogeneous material. Figure 4 shows the peak eluted from HPLC at a 40% isopropanol concentration.

The mol wt of the purified protease—32,000 kDa—(Fig. 3—insert) fully agrees with those of acid proteases from different species. After primary structure determination, the mol wt of chymosin and porcin pepsin were found to be 35,000 and 34,644, respectively (*12*). Molecular weights ranging between 30,000 and 38,000 were reported for fungal proteases (*5,13*). On the basis of their structural homology (*14*) and the molecular weight from those whose primary structure is already known, it is possible to affirm that polypeptide chains of acid proteases include 323–340 amino acid residues (*15*).

On the other hand, the N-terminal of these proteases is not conserved. Isoleucine or valine residues are located at this position in the pepsin group, whereas isoleucine or serine are in that of gastricsins (*16*). Proteases from *Mucor miehei* and *Penicillium janthinellum* have alanine as N-terminal, whereas that from *Mucor pusillus* has serine. Glycine—the N-terminal residue of the *Mucor bacilliformis* protease—is also present in that position in bovine and porcine chymosin.

Protease Characterization

Table 3 shows the amino acid composition of the purified protein, those from other fungal clotting proteases, and that of bovine chymosin. The *Mucor bacilliformis* protease is similar to that from *Mucor miehei,* which is used for cheese manufacture all over the world. It is important to point out the high carboxylate and hydroxylate residue contents; in addition, the total number of basic charges is very similar.

Table 3
Amino Acid Composition of *Mucor bacilliformis* Proteases Compared with Those from Other Mucor Strains and with Bovine Chymosin

	Mucor pusillus[a]	*Mucor miehei*[b]	*Mucor bacilliformis*[c]	Bovine chymosin[d]
Asp	44	42	45	31
Thr	21	32	40	21
Ser	22	38	35	26
Glu	20	25	22	30
Pro	14	19	15	12
Gly	34	37	48	24
Ala	16	29	24	11
Cys	2	4	4	7
Met	3	5	4	4
Val	24	27	28	22
Ile	12	20	20	17
Leu	15	21	29	19
Tyr	13	21	14	16
Phe	19	22	19	15
Lys	11	10	18	9
His	1	2	2	4
Arg	4	7	3	5
Trp	2	3	ND[e]	5

[a]Yu et al. (*17*).
[b]Rickert and Elliot (*13*).
[c]This work.
[d]Yu et al. (*17*).
[e]ND: not determined.

Figure 5 indicates that the protease shows maximum activity at pH 3.0 and 3.5 toward denatured hemoglobin and casein, respectively. Table 4 collects these data for different fungal proteases. Optimum pH values range between 2.0 and 4.0 and between 2.5 and 5.5 when hemoglobin and casein are used as substrate, respectively. On the other hand, clotting activity is retained after a 17-h incubation period at 4°C, in the 3–6 pH range; this behavior is identical to those of bovine chymosin (*20*) and the *Mucor miehei* protease (*21*). The *Mucor pusillus* enzyme is even more stable, since it is able to stand a 17-h incubation period at 4°C, in the 3–8 pH range. Protein stability against heat treatment was determined. As shown in Figure 6, a major clotting and proteolytic activity decrease is evidenced after heating a protease solution at 55°C for 30 min; under the same conditions, *Mucor miehei* protease retains over 90% of both activities as expected, since *Mucor bacilliformis* is a mesofilic strain, whereas *Mucor miehei* is a thermofilic one (Table 5).

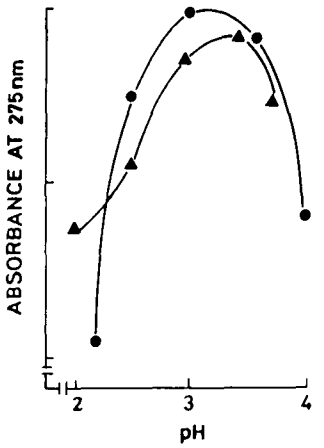

Fig. 5. pH effect on proteolytic activity of *Mucor bacilliformis* protease. Hemoglobin (●) and casein (▲) were used as substrates.

Table 4
Optimum pH Value for the Proteolytic Activity of Fungal Proteases

	Substrate	
	Haemoglobin	Casein
Endothia parasitica[a]	2.0	2.5
Mucor pusillus[b]	4.0	3.5
Irpex lacteus type A[c]	2.8	
Irpex lacteus type B[d]	2.8	
Mucor bacilliformis[e]	3.0	3.5

[a]Larson and Witaker (*18*),
[b]Arima et al. (*5*).
[c]Kobayashi et al. (*19*).
[d]Kobayashi et al. (*19*).
[e]This work.

Protease K_m for casein was determined. The value obtained—0.388 ± 0.076 g%—fully agrees with that reported for the *Mucor pusillus* enzyme—0.357 g% (*22*). The effect of PMSF, *p*-CMPS, and EDTA indicates that the protease is not metal dependent, nor does it possess serine or sulfhydryl active groups. However, the effect of pepstatin A, a specific acid protease inhibitor (23), leads to a clotting activity decrease that reaches 60% at a 1-μM final concentration (Table 6).

The above results allow inclusion of the *Mucor bacilliformis* protease in the aspartyl-protease family. On the other hand, its instability when submitted to heat treatment and its clotting/proteolytic activity ratio indicate its suitability for cheese manufacture.

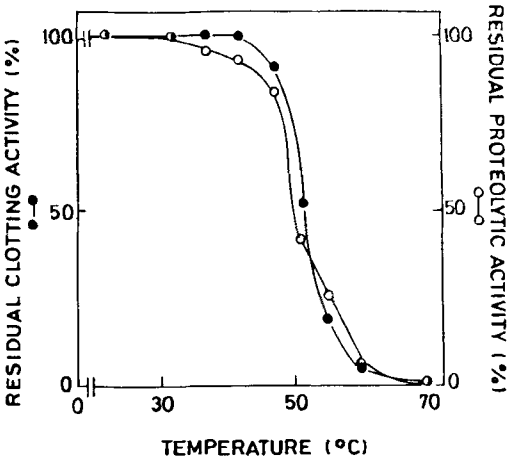

Fig. 6. Stability of the *Mucor bacilliformis* protease against heat treatment. Incubation period: 30 min.

Table 5
Heat Stability of *Mucor bacilliformis* and *Mucor miehei* Proteases[a]

	Residual proteolytic activity, %	
°C	*Mucor bacilliformis*	*Mucor miehei*
35	100	100
40	90	100
45	82	100
50	46	98
55	29	95
60	8	80

[a]Incubation period: 30 min.

Table 6
Pepstatin A Effect on *Mucor bacilliformis* Protease Clotting Activity

Pepstatin A concentration, μM	Residual clotting activity, (%)
0	100
10^{-3}	90
10^{-2}	80
10^{-1}	55
1	40
10	14
100	0

ACKNOWLEDGMENTS

We are indebted to Dr. Alejandro C. Paladini for his critical reading of the manuscript and to Dora M. Beatti for her excellent technical assistance. This work was supported in part by a CONICET grant.

REFERENCES

1. Dalgleish, D. G. (1987), in *Cheese: Chemistry, Physics and Microbiology*, vol. 1 (Fox, P. F., ed.), Elsevier, New York, pp. 63–95.
2. Drohse, H. B. and Foltmann, B. (1989), *Biochim. Biophys. Acta* **995,** 221.
3. Fraile, E. R., Muse, J. O., and Bernardinelli, S. E. (1981), *Eur. J. Appl. Microbiol. Biotechnol.* **13,** 191.
4. Thakur, M. S., Karanth, N. G., and Nand, K. (1990), *Appl. Microbiol. Biotechnol.* **32,** 409.
5. Arima, K., Yu, J., and Iwasaki, S. (1970), in *Methods of Enzymology*, vol. 19, Perlman, G. E. and Lorand, L., ed., Academic, New York, p. 446.
6. Food Chemical Codex (1981), Food and Nutrition Board. Division of Biological Sciences. Assemble of Life. Nat. Research Council. III ed. National Academic Press. Washington, D.C.
7. Laemmli, U. K. (1970), *Nature* **227,** 680.
8. Weber, K. and Osborn, M. (1969), *J. Biol. Chem.* **244,** 4406.
9. Shovers, J. and Fossum, G. (1972), *J. Dairy Sci.* **55,** 1532.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265.
11. Gray, W. R. (1972), in *Methods in Enzymology*, vol. 25B, Perlman, G. E. and Lorand, L., eds., Academic, New York, p. 121.
12. Tang, J., Sepulveda, P., Marciniszyn, J., Chen, K. C. S., Huang, W. Y., Tao, N., Liu, D., and Lanier, P. (1973), *Proc. Natl. Acad. Sci. USA* **70,** 3437.
13. Rickert, W. S. and Elliot, J. R. (1973), *Can. J. Biochem.* **51,** 1638.
14. Davies, D. R. (1990), *Ann. Rev. Biophys. Chem.* **19,** 189.
15. Blundell, T. L., Jenkins, J. A., Sewell, B. T., Pearl, L. H., Cooper, J. B., Tickle, I. J., Veerapandian, B., and Wood, S. P. (1990), *J. Mol. Biol.* **211,** 919.
16. Foltmann, B. (1987), in *Cheese, Chemistry, Physics and Microbiology, General Aspects*, vol. 1, Fox, P. F., ed., Elsevier Applied Sciences, London.
17. Yu, J., Tamura, G., and Arima, K. (1969), *Biochim. Biophys. Acta* **171,** 138.
18. Larson, M. K. and Whitaker, J. R. (1970), *J. Dairy Sci.* **53,** 253.
19. Kobayashi, H., Kusakabe, I., and Murakami, K. (1983), *Agric. Biol. Chem.* **47,** 551.
20. Foltman, B. (1966), *Compt. Rend. Triv. Lab. Carlsberg* **35,** 143.
21. Stenbert, M. Z. (1971), *J. Dairy Sci.* **54,** 159.
22. Somkuti, E. A. and Babel, F. J. (1968), *J. Bacteriol.* **95,** 1407.
23. Gilliland, G. L., Winborne, E. L., Nachman, J., and Wlodawer, A. (1990), *Proteins Struct. Funct. Genet.* **8,** 82.