APPLIED MICROBIOLOGY, Nov. 1968, p. 1727-1733

Vol. 16, No. 11
Printed in U.S.A.

Milk-clotting Enzyme from Microorganisms

V. Purification and Crystallization of *Mucor* rennin from *Mucor pusillus* var. *Lindt*[1]

KEI ARIMA, JUHYUN YU, SHINJIRO IWASAKI, AND GAKUZO TAMURA

Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Tokyo, Japan

Received for publication 5 June 1968

A rennin crystal was obtained from the crude milk-clotting enzyme of *Mucor pusillus* var. *Lindt*. The crude enzyme was purified by using columns of Amberlite CG-50, diethylaminoethyl Sephadex A-50, and Sephadex G-100. This purified enzyme was dissolved in 0.1 M sodium acetate (*p*H 5.0) buffer to a final concentration of 2 to 3%; ammonium sulfate (to 40% saturation) was added, and the resulting solution was placed in cellophane tubes. The enzyme solution was dialyzed against 0.1 M sodium acetate buffer (*p*H 5) containing ammonium sulfate was added dropwise to the outside solution of the cellophane tube, and the concentration of ammonium sulfate in the cellophane tube increased gradually. The crystals of enzyme were formed in the cellophane tube when the concentration reached approximately 50% saturation. After the enzyme solution was concentrated in the freezer, the crystals were obtained. The activity of the crystalline enzyme was inhibited by Hg^{2+}, Ag^{+}, Zn^{2+}, and $KMnO_4$.

The milk-clotting enzyme produced by *Mucor pusillus* var. *Lindt* and its properties were investigated by Arima and others by using a highly purified enzyme preparation (1–4, 6, 7). Recently, the enzyme was purified to a higher degree, and was successfully crystallized from the crude enzyme. The present paper describes the method of crystallization and some properties of the crystalline enzyme.

MATERIALS AND METHODS

Crude enzyme. The crude enzyme preparation obtained from the culture of *M. pusillus* var. *Lindt* on wheat bran was the kind gift of the Meito Co. Ltd., Japan.

Determination of milk-clotting activity. To 0.5 ml of the enzyme solution in a 25-ml tube, 5 ml of 10% (w/w) skim milk powder (Snow Milk Products Co. Ltd., Japan) in 0.01 M calcium chloride solution was added. The tube was incubated at 35 C. The milk-clotting activity was detected as described previously (3). The enzyme amount which clotted the substrate within 1 min was defined as 400 units.

Column chromatography. The crude enzyme was purified by using columns of Amberlite CG-50, diethylaminoethyl (DEAE) Sephadex A-50, and Sephadex G-100. Sodium acetate buffer (*p*H 3.5 to 5.0) was used for these experiments. The procedure with Amberlite CG-50 and DEAE Sephadex A-25 was described by Iwasaki, Tamura, and Arima (4), but we modified their method to some extent in this experiment.

Determination of proteolytic activity. The proteolytic activity was measured by a modification of Anson's (2) method. To 2.5 ml of 0.5 to 1.5 (w/w) casein solution (Hammarsten) in 0.02 M potassium phosphate buffer (*p*H 6.5) was added 0.5 ml of the enzyme solution, and the mixture was incubated at 35 C for 10 min. After incubation, 2.5 ml of 0.44 M trichloroacetic acid was added; then the mixture was filtered. One milliliter of three times diluted Folin reagent and 2.5 ml of 0.55 M sodium carbonate were added to 1 ml of the filtrate. After the solution was kept at 35 C for 20 min, optical density (OD) at 660 nm was measured with a Hitachi spectrophotometer.

RESULTS

Purification and crystallization of clotting enzyme. The crude enzyme was dissolved in 5 liters of tap water, and the solution (OD 280 nm, 1.134×10^5 and 5.2×10^7 units) was adjusted to *p*H 3.5 with 1 N HCl. The enzyme solution was adsorbed on an Amberlite CG-50 column (10 by 60 cm). The fraction containing the pigment was eluted with 150 liters of 0.05 M sodium acetate buffer (*p*H 3.5) as shown in Fig. 1. The fraction with milk-clotting activity was eluted with sodium acetate buffer (*p*H 5.0) and collected. The total volume of the enzyme eluate was 15.0 liters. The activity was 5.1×10^7 units (total OD at 280 nm,

[1] Presented at the Annual Meeting of the Agricultural Chemical Society of Japan at Tokyo, April 1968, and at the 3rd Meeting of the Corporation of Japan Appl. Enzyme at Osaka, December 1967.

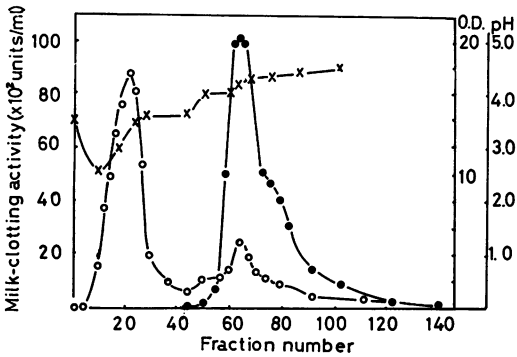

FIG. 1. *Column chromatogram of Mucor rennin on Amberlite CG-50 (10 by 60 cm). Milk-clotting activity, ●; OD 280 nm, ○; pH of the solution, ×; eluted volume, 300 to 330 ml, pH of 0.05 M sodium acetate buffer; fractions 1 to 50, pH 3.5; fractions 51 and above, pH 5.0.*

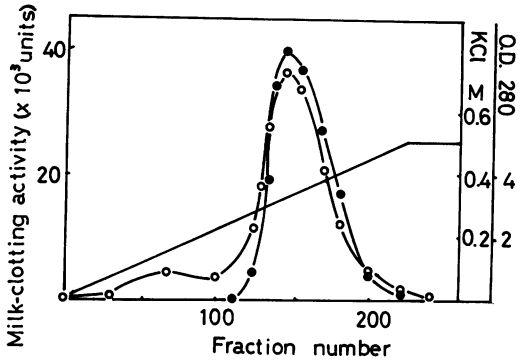

FIG. 2. *Chromatographic pattern of Mucor rennin on DEAE Sephadex A-50 (6 by 24 cm). Milk-clotting activity, ●; OD 280 nm, ○; concentration of KCl, unbroken line; elution buffer, 0.05 M sodium acetate buffer (pH 5.0); eluted volume, 20 ml.*

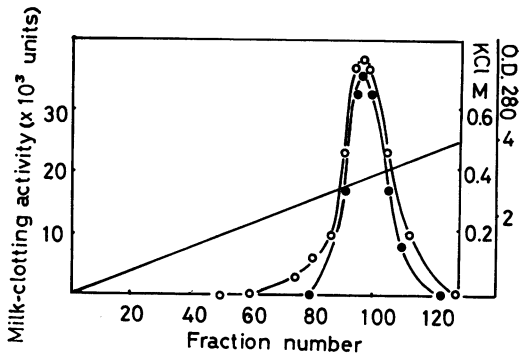

FIG. 3. *Chromatographic pattern of Mucor rennin on DEAE Sephadex A-50 (4 by 29 cm). Milk-clotting activity, ●; OD 280 nm, ○; concentration of KCl, unbroken line; elution buffer, 0.05 M sodium acetate buffer (pH 5.0); elution volume, 20 ml.*

24 × 10^3; specific activity, 2,140 units). The eluate was adjusted to *p*H 3.5 with 1 N HCl. The eluted solution was again adsorbed on an Amberlite CG-50 column which had been equilibrated with 0.05 M sodium acetate buffer, *p*H 3.5. By this rechromatography, 16.5 liters of enzyme solution of specific activity 2,820 units (OD at 280 nm; 14 × 10^3) was obtained; the total activity was 4.024 × 10^7 units.

The enzyme solution was then adsorbed on a DEAE Sephadex A-50 column (6 by 24 cm) equilibrated with 0.05 M sodium acetate buffer (*p*H 5.0). The elution was carried out with a linear gradient beginning without KCl and ending with 0.5 M HCl. The reservoir contained 1 liter of 0.05 M sodium acetate buffer (*p*H 5.0) and 0.5 M KCl, and the mixing chamber contained 1 liter of the same buffer without potassium chloride. The typical chromatographic pattern is shown in Fig. 2. The protein peak with milk-clotting activity appeared to be eluted between 0.30 and 0.45 M KCl. The active fraction was collected, and the concentration of KCl in the enzyme solution was diluted to less than 0.2 M by adding 0.05 M sodium acetate buffer (*p*H 5.0). The solution was then passed through a new DEAE Sephadex A-50 column which had been equilibrated with the same buffer. By this rechromatography on Sephadex A-50, 780 ml of the enzyme solution was obtained (Fig. 3).

Ammonium sulfate was added to the enzyme solution to 70% saturation. The solution was allowed to stand at 5 C for 10 hr. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min at 0 C. The sedimented material was dissolved in a minimal amount of 0.05 M sodium acetate (*p*H 5.0). The solution was then fractionated by gel filtration by use of a Sephadex G-100 column and 0.05 M sodium acetate buffer (*p*H 5.0). By this procedure, part of the pigment fraction was removed (Fig. 4). The remaining pigment was removed by repeated gel filtration on Sephadex G-100. The active fractions (20.31 × 10^6 units) were collected and ammonium sulfate was added slowly to 70% saturation. After 10 hr at 0 C, the precipitate was collected by centrifugation and was dissolved in 0.1 M sodium acetate buffer (*p*H 5.0) to give a final concentration of 2 to 3% protein. Saturated ammonium sulfate solution was added slowly with gentle stirring until a slight turbidity formed. The precipitate was removed by centrifugation and discarded. The ammonium sulfate concentration was slowly increased to 50% saturation with saturated ammonium sulfate. The supernatant fluid was put into a flask and

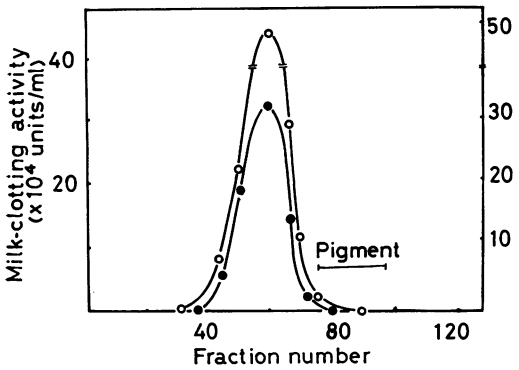

FIG. 4. *Column chromatogram of Mucor rennin on Sephadex G-100 (3 by 8 cm). Milk-clotting activity, ●; OD 280 nm, ○; elution buffer, 0.05 M sodium acetate buffer (pH 5.0).*

allowed to stand at 3 to 4 C in a refrigerator for 2 months. Crystallization started when the concentrations of ammonium sulfate and enzyme increased slowly by naturally occurring evaporation. Crystallization could be greatly hastened by seeding enzyme crystals into the concentrated solution. By this seeding procedure, the crystallization started in 10 to 15 hr. The different forms of crystals were observed as shown in Fig. 5. All of these crystalline forms have the same specific activity.

It may be that the different forms of crystals reported result from the different methods employed for crystallization. Thus, it took about 2 months to obtain the elliptical shape by natural evaporation and crystallization in a refrigerator. The other forms of the crystals were obtained by the dialyzation-crystallization method.

Another method of crystallization was as follows. A 2 to 3% enzyme solution was put into a cellophane tube, and the tube was dialyzed against 0.1 M sodium acetate buffer containing 50% saturated ammonium sulfate. A saturated solution of the ammonium sulfate was added dropwise to the outside solution to give a final saturation of 70%. The concentration of ammonium sulfate in the cellophane tube slowly increased and finally the crystals of the enzyme formed. The procedures for this purification process are summarized in Fig. 6 and Table 1.

The crystals of the enzymes were separated by centrifugation at 10,000 × *g* for 10 min and were dissolved in distilled water. The solution was evaporated to dryness by freeze-drying after the enzyme solution had been dialyzed against distilled water for 24 hr. The dry weight of enzyme obtained was 2.35 g, which corresponded to 2,580 OD units at 280 nm. Total milk-clotting activity was 172 × 10^7 units, and specific activity was 6,670 units of milk-clotting activity per OD unit at 280 nm.

Sedimentation behavior and electrophoretic pattern of crystalline enzyme. The homogeneity of crystalline enzyme in 0.1 M sodium acetate buffer (*p*H 5.0) was examined by both Tiselius electrophoresis and ultracentrifugal analysis. Tiselius electrophoresis was carried out at 90 v and 5 ma. The enzyme migrated essentially as a single symmetrical peak. The ultracentrifugal analysis was carried out at 55,400 rev/min at 11.3 C for 3 hr and 17 min. The sedimentation pattern also showed a symmetrical peak. From these results (Figs. 7 and 8) it was confirmed that the crystalline enzyme was homogeneous.

Ultraviolet spectrum. The spectrum of *Mucor* rennin crystals which were dissolved in the distilled water is shown in Fig. 9. The maximal absorption was given at 276 nm, and the specific extinction of 1.0% (w/w) (*Mucor* rennin) solution at 280 nm ($E_{1\%}^{1\,cm}$) was 10.

Ratio of milk-clotting activity of Mucor rennin. The ratio of the milk-clotting activity of Mucor rennin crystals to their proteolytic activity was compared with those of other proteases. The value of the ratio was expressed as milk-clotting units per OD unit at 660 nm (proteolytic activity). The

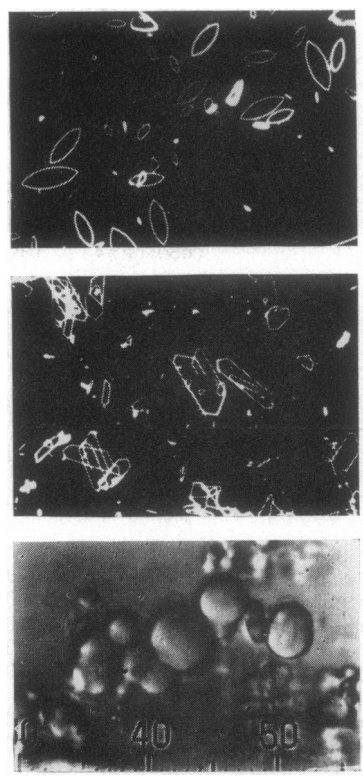

FIG. 5. *Different forms of crystalline Mucor rennin.*

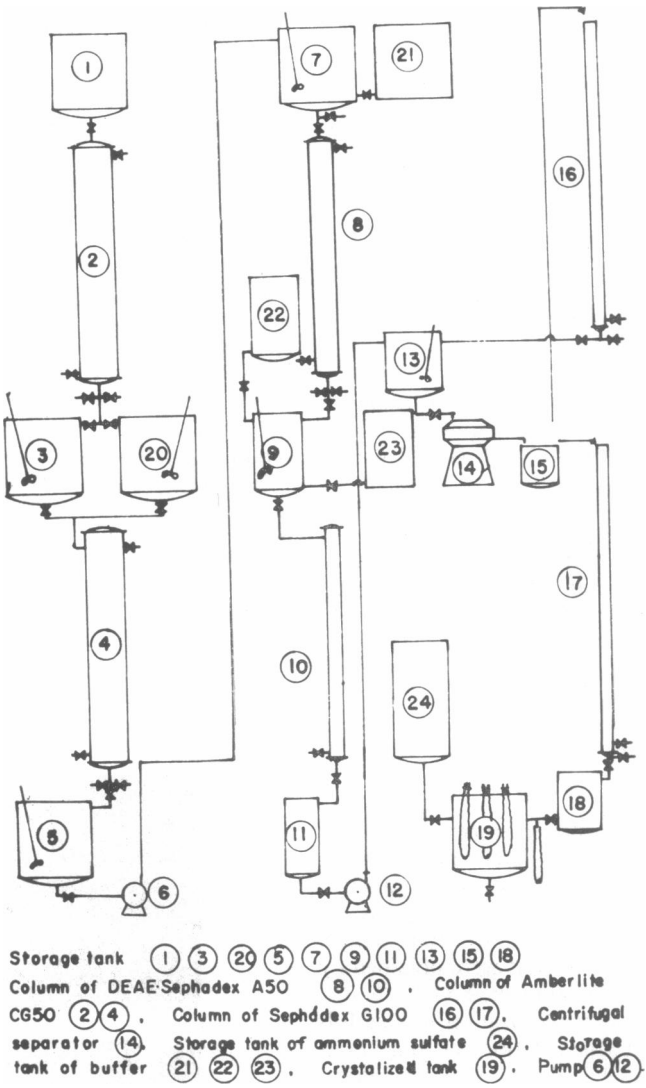

FIG. 6. *Purification and crystallization process of Mucor rennin.*

TABLE 1. *Purification process of Mucor rennin crystal*

Determination	OD 280 nm		Clotting activity		Specific activity	
	OD 280 nm	Yield	× 10^3 Units	Yield	OD 280 nm	Yield
		%		%		
Crude enzyme	113,400	100	52,000	100	460	1.0
Amberlite CG-50	24,000	21.5	51,000	99.3	2,140	4.7
Amberlite CG-50	14,000	12.4	40,240	77.3	2,870	6.2
DEAE Sephadex A-50	6,660	5.9	29,667	57.2	4,450	9.1
DEAE Sephadex A-50 $(NH_4)_2SO_4$, saturated 0.7	4,220	3.7	25,480	49.0	6,040	13.2
Sephadex G-100	3,850	3.4	23,320	44.8	6,050	13.3
Sephadex G-100 $(NH_4)_2SO_4$	3,050	3.0	20,310	38.7	6,650	14.4
Mucor rennin crystal, 2.35 g	2,910	2.6	19,300	37.1	6,670	14.4
	2,580	2.6	17,200	33.1	6,670	14.5

ratio of rennin was 7,350 units per OD unit at 660 nm, and that of Mucor rennin crystals was 4,650 units per OD unit at 660 nm, whereas Pfizer microbial rennin was 2,590 units per OD unit at 660 nm. Molsin (Kikkoman Co. Ltd.), ficin, trypsin, pepsin, papain, and Biodiastase (Amano Co. Ltd.) were less than 393 units per OD unit at 660 nm (Table 2).

Influence of metal ion and other chemicals. The final concentration of chemicals added to the enzyme solution (Mucor rennin crystal, 9.5 mg/ml) was adjusted to 10^{-3} M for metals, and 10^{-2} M for other test reagents. After the enzyme-chemical mixture was kept for 30 min at 35 C, proteolytic and milk-clotting activities were measured (Tables 3 and 4). The metals ions of Ag, Zn, and Hg reduced the proteolytic activity by more than 30% of the control, whereas Cu and Mn activated the proteolytic activity. $KMnO_4$ inhibited the milk-clotting activity strongly; other chemicals had only a slight effect. The activity of the purified enzyme is inhibited by Hg and Fe ions (3).

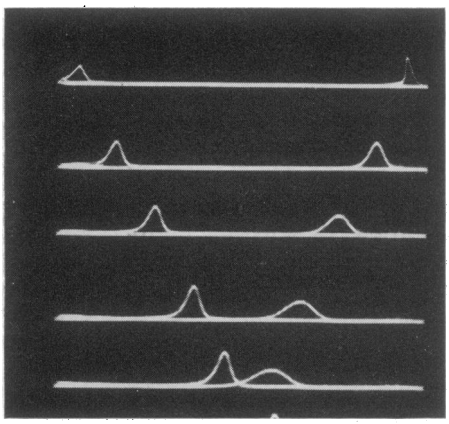

FIG. 7. *Electrophoretic patterns of Mucor rennin crystal. Mucor rennin solution, 2% in 0.1 M sodium acetate buffer (pH 5.0); 5 ma, 71 to 90 v. The values of the patterns were as follows (from top to bottom): 0 min, 10 C; 15 min, 20 C; 30 min, 20 C; 45 min, 25 C; 57 min, 30 C.*

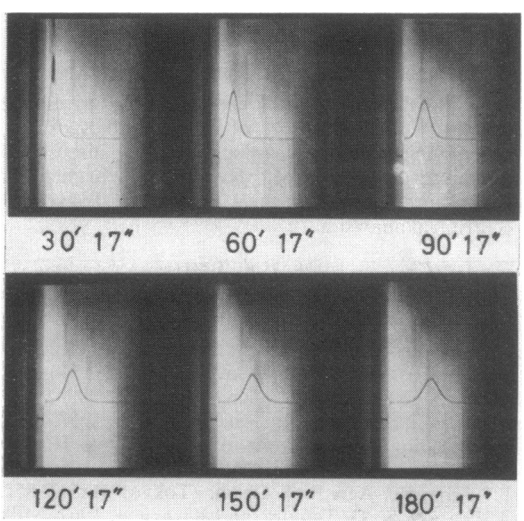

FIG. 8. *Ultracentrifuge patterns of Mucor rennin crystal. Concentration of Mucor rennin solution in 0.1 M sodium acetate buffer (pH 5.0); 1.0% Mucor rennin solution. Condition of ultracentrifugation, 55,400 rev/min, 11.3 C.*

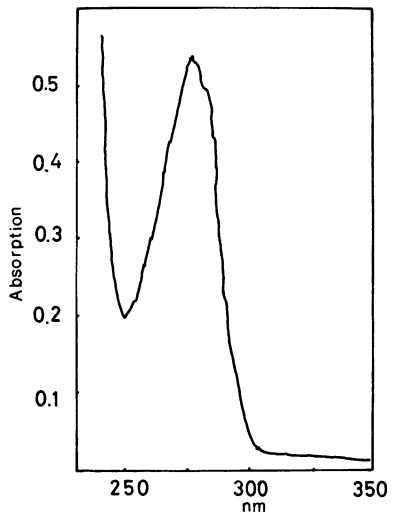

FIG. 9. *Ultraviolet spectrum of Mucor rennin crystal. Mucor rennin crystal was dissolved with 0.1 M sodium acetate buffer (pH 5.0) solution.*

TABLE 2. *Ratio of clotting activity to proteolytic activity of various proteases*

Protease	Clotting activity (units/ml)	Proteolytic activity (OD 660 nm)	Ratio (units/OD 660 nm)
Rennin	293	0.04	7,350
Mucor rennin crystal	511	0.11	4,650
Pfizer microbial rennin	750	0.29	2,590
Papain	216	0.59	367
Pepsin	2	0.015	133
Trypsin	1.6	0.44	4
Molsin	1.3	0.18	7
Ficin	267	0.68	393
Biodiastase	115	0.83	138

DISCUSSION

It has been reported (4) that the crude enzyme was highly purified by column chromatography

TABLE 3. *Influence of metals on milk-coagulation activity of Mucor rennin crystal*[a]

Metal	Proteolytic activity
	%
$AgNO_3$	54
$NiCl_2$	—
$MgCl_2$	100
$SnCl_2$	98
$CdCl_2$	85
$Pb(NO_3)_2$	80
$ZnSO_4$	50
$CuSO_4$	—
$HgCl_2$	68
$BaCl_2$	105
$MnSO_4$	129
$CaCl_2$	102
NaCl	104
None	100

[a] Final concentration of metals in Mucor rennin crystal solution (9.5 mg/ml) was 10^{-3} M. After the mixture was kept for 30 min at 35 C, the activity was measured with the mixture.

TABLE 4. *Influence of chemicals on milk-clotting activity*[a]

Chemical	Residual coagulation activity
	%
$K_3[Fe(CN)_6]$	85
8-Hydroxyquinoline	89
Iodoacetic acid	105
Sodium citrate	93
Sodium oxalate	94
Sodium thiosulfate dipyridyl	99
L-Cysteine HCl	118
Ethylenediaminetetraacetate	119
p-Chloromercuribenzoic acid	100
L-Ascorbic acid	116
$KMnO_4$	0
None	100

[a] Final concentration of chemicals in Mucor rennin solution (9.5 mg/ml) was 10^{-2} M. After the mixture was kept for 30 min at 35 C, the activity was measured with the mixture.

on Amberlite CG-50 and DEAE Sephadex A-25. However, the crystalline enzyme had not been obtained. Efforts were made to successfully crystallize the enzyme on Amberlite CG-50 and DEAE Sephadex G-100 columns. The crystalline enzyme had a specific activity of 6,850 units (Table 1).

In a previous investigation (4), the enzyme solution was concentrated by ammonium sulfate before charging on the column. In the present method, we were able to use the crude solution directly. The enzyme was adsorbed on Amberlite CG-50 column when the *p*H of the enzyme solution was adjusted to 3.5. An active fraction was eluted in the range of 0.30 to 0.45 M KCl from DEAE Sephadex A-50 (Fig. 2). Two liters of the concentrated enzyme was obtained from 16.5 liters of initial crude enzyme by column chromatography (Fig. 3). When the concentration of KCl in the enzyme solution was adjusted to less than 0.2 M, an active fraction was adsorbed on DEAE Sephadex A-50. Rechromatography on DEAE Sephadex G-100 was effective for purification of the enzyme. The crystallization method with the cellophane tube brought about good recovery of Mucor rennin crystals.

Tsugo et al. (6) reported that in their experiments of making Camembert, Gouda, and cottage-type cheese, the yield of the cheese by our crude microbial rennet was almost the same as that by animal rennet or even a little higher. Richardson, Nelson, and Lubnow (5) reported that the crude enzyme produces a slightly more nonprotein nitrogen than through the time course. From Table 2, if we assume that the rate of milk-clotting activity of rennin to its proteolytic activity (units per OD unit at 960 nm) is 100%, the rate of Mucor rennin crystal is 63%. From these results we can say that the value or the ratio (clotting activity per proteolytic activity) of *Mucor* rennin crystal is closer to that of animal rennin than is any other milk-clotting protease thus far reported. These results and the feasibility of industrial-scale production strongly support the concept that *Mucor* rennin will be useful for the manufacture of cheese, as a rennin substitute.

ACKNOWLEDGMENT

We thank to K. Yano, Department of Agricultural Chemistry, the University of Tokyo, and K. Yasui, Tokyo University of Education, for helpful discussions during this work, and K. Kaguma, Department of Agricultural Chemistry, the University of Tokyo, for centrifugal analysis.

LITERATURE CITED

1. Arima, K., S. Iwasaki, and G. Tamura. 1967. Milk clotting enzyme from microorganisms. I. Screening test and the identification of the potent fungus. Agr. Biol. Chem. (Tokyo) **31**:540–545.
2. Anson, M. L. 1938. J. Gen. Physiol. **22**:79.
3. Iwasaki, S., G. Tamura, and K. Arima. 1967. Milk clotting enzyme from microorganisms. II. The enzyme production and properties of crude enzyme. Agr. Biol. Chem. (Tokyo) **31**:547–551.
4. Iwasaki, S., G. Tamura, and K. Arima. 1967. Milk clotting enzyme from microorganisms. III. Purification of enzyme and its properties. Agr. Biol. Chem. (Tokyo) **31**:1421–1426.
5. Richardson, G. H., J. H. Nelson, and R. E. Lub-

now. 1967. Rennin like enzyme from *Mucor pusillus* for cheese manufacture. J. Dairy Sci. **50**:1066–1072.
6. Tsugo, T., K. Kokichi, U. Yoshino, A. Ozawa, and K. Arima. 1964. Studies on cheese making by using milk clotting enzyme of *Mucor pusillus Lindt*. II. Cheese making experiment. Japan. J. Zootech. Sci. **35**:229–237.
7. Yu, J., G. Tamura, and K. Arima. 1968. Studies on active site of Mucor rennin and reaction of Ca ion on milk coagulation. Agr. Biol. Chem. **32**: 1048–1050.