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MILK-CLOTTING ENZYME FROM MICROORGANISMS

VI. PROPERTIES OF CRYSTALLINE MILK-CLOTTING ENZYME (MUCOR RENNIN) ISOLATED FROM *MUCOR PUSILLUS* VAR. LINDT

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SUMMARY

The crystalline milk-clotting enzyme isolated from *Mucor pusillus* var. Lindt, has an acid protease activity. The optimum pH for the digestion of κ-casein is 4.5, while that for hemoglobin digestion is 4.0.

The physical properties of the crystalline enzyme are as follows: partial specific volume is 0.74; sedimentation coefficient is $2.39 \cdot 10^{-13}$ ($cm \cdot g \cdot sec^{-1} \cdot dyne^{-1}$); diffusion coefficient, $7.9 \cdot 10^{-7}$ $cm^2 \cdot sec^{-1}$; frictional coefficient (f/f_0), 1.33. Molecular weight was given as 29 000 by Svedverg's method and 30 600 by Yphantis' method. The number of amino acid residues contained in 1 mole of protein of the crystalline enzyme is 277–281 ($(Cys)_2$, Met_3, Asp_{44}, Thr_{21}, Ser_{22}, Glu_{20}, Pro_{14}, Gly_{34}, Ala_{16-17}, Val_{24}, Ile_{12}, Leu_{15}, Tyr_{13}, Phe_{19}, His_{1-2}, Lys_{11-12}, Arg_4, Trp_{2-3}).

INTRODUCTION

Many milk coagulants of microbial origin have been investigated during the past years[4,7,8,15,18–20,23,24]. KNIGHT[16] tested numerous molds for rennin-like enzyme production and finally *Byssochlamys fulva* was selected. *Enthodia parasitica* has also been selected as a useful fungus for the production of a rennin-like enzyme[9].

A crude enzyme[2,3,13,14,30] having rennin-like, milk-clotting activity is also produced by *Mucor pusillus* var. Lindt. We obtained the crystalline milk-clotting enzyme from the crude enzyme of *M. pusillus* var. Lindt[26–28] and the crystalline enzyme was given the name Mucor rennin. This paper describes the results of various experiments on the properties of the crystalline enzyme.

MATERIALS AND METHODS

Crystalline Mucor rennin

The crystalline enzyme has been obtained from the crude enzyme of *M. pusillus* var. Lindt by the procedures of purification and crystallization as follows[26–28]. The

crude enzyme was purified by the procedures using the columns of Amberlite CG-50, DEAE-Sephadex A-50 and Sephadex G-100. This purified enzyme was dissolved in 0.1 M sodium acetate buffer (pH 5.0) to make its concentration 2–3%. To this solution was added $(NH_4)_2SO_4$ to 40% saturation, and then this solution was put into a cellophane tube. The enzyme solution in the tube was dialysed against 0.1 M sodium acetate buffer (pH 5.0), containing $(NH_4)_2SO_4$ of 40% saturation. $(NH_4)_2SO_4$ was added to the outside solution of the cellophane tube dropwise to increase the concentration in the tube gradually. The crystals of enzyme were formed in the cellophane tube when the concentration reached approx. 50% saturation. After the enzyme solution was concentrated in a refrigerator, the crystals were obtained.

The crystalline enzyme (1.0 g) was dissolved in 10 ml of distilled water. The solution was evaporated to dryness by the freeze-drying method after the enzyme solution was dialysed against 200 vol. of distilled water for 24 h. This dried enzyme was used for the experiment.

Determination of milk-clotting activity[27]

To 0.5 ml of the enzyme solution in a tube of 25 ml volume, were added 5 ml of 10% (w/w) skim milk powder (Snow Brand Milk Products Co. Ltd.) in 0.01 M $CaCl_2$ solution and the tube was incubated at 35°. The milk-clotting activity was measured as described previously. The amount of enzyme which clotted 5 ml of the substrate within 1 min was defined as 400 units.

Determination of proteolytic activity

The proteolytic activity was measured by the modified ANSON's method[1]: a mixture of 2.5 ml of 0.5–1.5% (w/w) Hammarsten casein solutions and 0.02 ml of the enzyme solution was incubated at 35° for 10 min. After incubation, 2.5 ml of 0.44 M trichloroacetic acid solution were added to the solution, and then the mixture was filtrated. 1 ml of 3-times-diluted Folin reagent and 2.5 ml of 0.55 M Na_2CO_3 were added to 1 ml of the filtrate. After the solution was kept at 35° for 20 min, the absorbance at 660 mμ was measured using a Hitachi spectrophotometer.

Measurement of viscosity, infrared spectrum and ultraviolet spectrum

The viscosity of the Mucor rennin crystal solution, buffer and water was measured respectively with an Ostwald viscosimeter. The infrared spectrum was measured by a Koken DS–31 spectrometer. An ultraviolet spectrum determination was run with a JNM–4H–100 spectrum.

Ultracentrifugal pattern and diffusion pattern

Analytical ultracentrifugation was carried out with a Hitachi analytical ultracentrifugal UCA–1A apparatus. The diffusion coefficient was measured with a Hitachi Tiselius diffusion cell.

Analysis of amino acids

Analysis of amino acids was carried out with a Shimazu auto-analyzer. Mucor rennin crystal, 4.04 mg, was hydrolyzed in 0.08 ml of 6 M HCl at 110° for 24 h. HCl was removed under vacuum. The volume of residual solution was adjusted to 5 ml by adding buffer solution (pH 2.2). 50 μl of the solution were used for the analysis

of amino acid. The estimation of Ser and Thr was not corrected. The amount of Trp was determined by ultraviolet absorption[12].

On the analysis of Met and Cys of Mucor rennin, the oxidized Mucor rennin was obtained by the SCHRAM preparation method[22]. To 1.85 mg of the oxidized Mucor rennin, 0.366 ml of 6 M HCl solution was added and the mixture was kept at 110° for 24 h. The solution was adjusted to a final volume of 0.5 ml with distilled water. 50 μl of the solution was used for the analysis of methionine and cysteine in Mucor rennin.

RESULTS

Properties of Mucor rennin crystal

Optimum pH for proteolytic activity. To 5.0 ml of 0.5% (w/w) substrate or hemoglobin dissolved in 0.05 M sodium acetate buffer (pH 1.0–5.0) or McIlvaine buffer (pH 5.0–8.0), 0.5 ml of the enzyme solution was added. The solution was then incubated for 10 min to allow for the decomposition of substrate. Optimum pH was 4.5 for κ-casein and 4.0 for hemoglobin, respectively. Optimum pH for Hammarsten casein is around 3.5 (ref. 13). These results show that the optimum pH of Mucor rennin crystal is similar to that of animal rennin[21,29].

Effect of pH on milk coagulation. The skim milk solution was adjusted to various pH's (between pH 5.0 and 7.0) with 1 M HCl and 1 M NaOH solution so that the effect of pH on milk coagulation could be investigated. The milk solution clotted most rapidly at pH 5.5. At pH 7.0 the milk solution did not clot within 30 min. Similar results were obtained with the crude enzyme[31,13]. Rennet activity[31] proved weaker in alkaline conditions than in acidic conditions.

Physical properties and amino acid composition of Mucor rennin crystal

Sedimentation, diffusion and frictional coefficients. The sedimentation coefficient of the purified enzyme[32] has been reported but the value was not corrected into the standard state ($s^\circ_{20,w}$). Therefore, the concentration of Mucor rennin crystal was adjusted to different levels with this buffer and then the Mucor rennin solutions were sedimented. The results are given in Fig. 1. The value of the sedimentation coefficient at 19.2° was given as $2.25 \cdot 10^{-13}$ (cm·g·sec^{-1}·dyne^{-1}). The density of 0.75% Mucor rennin solution was 1.0103. Partial specific volume of Mucor rennin crystal was

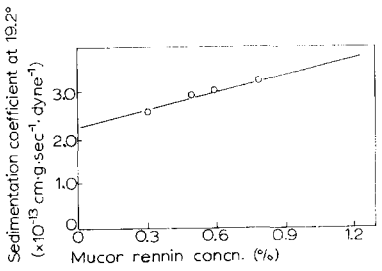

Fig. 1. Determination of sedimentation coefficient of Mucor rennin crystal. Mucor rennin crystal was dissolved in 0.1 M sodium acetate buffer (pH 5.0) solution containing 0.1 M KCl to give a final concentration of 0.3, 0.5, 0.6, and 0.8% (w/v), respectively. The sedimentation coefficients at 19.2° of the enzyme solutions were determined.

obtained as 0.74 from these values. The observed sedimentation coefficient at 19.2° was corrected into the standard state ($s°_{20,w}$) and the value was given as $2.39 \cdot 10^{-13}$ ($cm \cdot g \cdot sec^{-1} \cdot dyne^{-1}$).

1.5% Mucor rennin solution was used for the diffusion experiment. The typical schlieren pattern by the diffusion of Mucor rennin crystal is shown in Fig. 2. The diffusion coefficient was calculated from both equations by the maximum ordinate

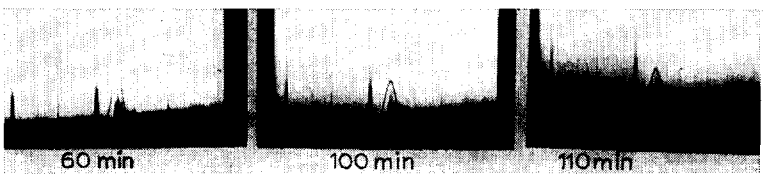

Fig. 2. Diffusion patterns of Mucor rennin crystal. Mucor rennin crystal was dissolved in 0.1 M sodium acetate buffer (pH 5.0) containing 0.1 M KCl to give a final concentration of 1.5% (w/w). The photographs were taken after 60, 100 and 110 min of diffusion at 28.5°.

method and the maximum ordinate area method[17]. The value of diffusion coefficients at 28.0 and 28.5° are respectively indicated as $9.35 \cdot 10^{-7}$ ($cm^2 \cdot sec^{-1}$) and $10.23 \cdot 10^{-7}$ ($cm^2 \cdot sec^{-1}$) in Fig. 3. Correction of the measured value for the standard condition ($D_{20,w}$) was performed. The value of $D_{20,w}$ was given as $(7.9 \pm 0.3) \cdot 10^{-7}$ ($cm^2 \cdot sec^{-1}$). The frictional ratio (f/f_0) was 1.33.

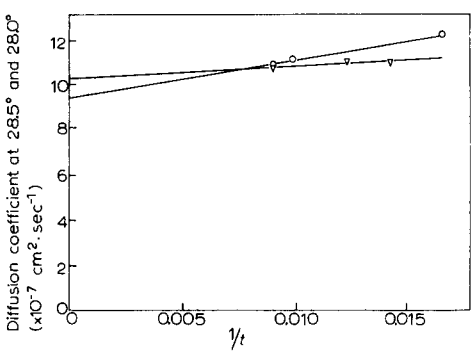

Fig. 3. Relative of diffusion coefficient and diffusion time. Diffusion coefficient of Mucor rennin crystal at 28.5° (○) and at 28.0° (▽).

Molecular weight

Values for the sedimentation coefficient, diffusion coefficient, partial specific volume and density obtained from the above results were used in Svedberg equation[25]. The molecular weight of the Mucor rennin crystal was calculated roughly as 29 000 ± 1400. The measurement of molecular weight was carried out by YPHANTIS' method[6]. The apparent molecular weight of Mucor rennin crystal was estimated as 30 600 from the schlieren pattern of Fig. 4.

Amino acid composition of Mucor rennin crystal

The results of the amino acid analysis of Mucor rennin crystal are given in

Fig. 4. Schlieren patterns of Mucor rennin crystal by Yphantis' method. Mucor rennin crystal was dissolved with 0.1 M sodium acetate buffer (pH 5.0) solution containing 0.1 M KCl. The photographs were taken (a) after 14 min 15 sec sedimentation at 14 400 rev./min, 12.3° and 80° (○), and (b) after 80 min 45 sec sedimentation at 11 600 rev./min and 11.0°.

Table I. The number of amino acid residues contained in 1 mole of protein of Mucor rennin crystal was 277-281 ($(Cys)_2$, Met_3, Asp_{44}, Thr_{21}, Ser_{22}, Glu_{20}, Pro_{14}, Gly_{34}, Ala_{16-17}, Val_{24}, Ile_{12}, Leu_{15}, Tyr_{13}, Phe_{19}, His_{1-2}, Lys_{11-12}, Arg_4, and Trp_{2-3}). Chemical elemental analysis of Mucor rennin crystal is summarized in Table II. The experimental values were as follows: C, 49.24 and 49.59%; H, 6.99 and 7.44%; N, 13.86 and 14.19%; S, 0.57 and 0.65%. These values agree with the theoretical values obtained from amino acid composition and NH_3.

DISCUSSION

From the investigation on the properties of protein in the Mucor rennin crystal, the following results were obtained: partial specific volume is 0.74; sedimentation coefficient is $2.39 \cdot 10^{-13}$ ($cm \cdot g \cdot sec^{-1} \cdot dyne^{-1}$); diffusion coefficient is $(7.9 \pm 0.3) \cdot$

TABLE I

AMINO ACID COMPOSITION OF MICROBIAL RENNIN, PRORENNIN AND RENNIN

Amino acid	*Microbial rennin*		*Rennin**	*Prorennin**
	Mol. wt. 30 600	*Residues*	*Mol. wt. 31 100*	*Mol. wt. 36 200*
$^1/_2$-Cys	2.31	2		
Met	3.17	3	7	7
Asp	43.6	44	31	33
Thr	21.2	21	28	21
Ser	22.1	22	27	31
Glu	19.7	20	29	36
Pro	14.1	14	12	14
Gly	33.5	34	25	29
Ala	16.4	16-17	13	15
Val	23.8	24	21	23
Ile	11.8	12	15	19
Leu	14.8	15	19	26
Tyr	12.8	13	15	18
Phe	18.9	19	14	14
His	1.42	1-2	4	5
Lys	11.4	11-12	8	13
Arg	4.1	4	7	5
Trp	2.45	2-3		
	277.52	277-281	263	313
NH_3	14.3			

* Ref. 11.

TABLE II

ELEMENTARY ANALYSIS OF MUCOR RENNIN CRYSTAL

	C (%)	*H* (%)	*N* (%)	*S* (%)
Theoretical values			14.48	0.53
Analytical values	49.24	6.99	13.86	0.65
	49.59	7.44	14.19	0.57

10^{-13} $cm^2 \cdot sec^{-1}$; the ratio of frictional coefficient is 1.33. The molecular weight of the Mucor rennin crystal, is indicated as 29 000 ± 1400 by Svedberg's method and 30 600 by Yphantis' method. Thus, the molecular weight is roughly estimated to be 29 800. The number of amino acid residues contained in 1 mole Mucor rennin crystal is 277–281.

The optimum pH is 4.5 for κ-casein and 4.0 for hemoglobin, respectively. The thermal stable pH range is from pH 4.0 to 6.0 and the enzyme is most stable at pH 5.0.

SCHWANDER, ZAHLER AND NITSCHMAN[21] have reported on the physical properties as shown in Table III and FOLTMAN AND HARTLEY[10,11] on the amino acid composition of rennin. The optimum pH for hydrolysis of rennin is 3.7 for casein[22] and hemoglobin[5].

The general properties of Mucor rennet[2,3,13,14,30,31] and the Mucor rennin crystal differ slightly from those of animal rennet.

Although the authors have found the structure of Mucor rennin crystal to be different from that of rennin, the Mucor rennin crystal is available for the manufacture of cheese.

TABLE III

SUMMARIZED PHYSICAL PROPERTIES OF RENNIN AND MUCOR RENNIN

Property		*Value*		
		Mucor rennin	*Rennin**	*Prorennin**
Frictional coefficient	f/f_0	1.33	0.98**	
Sedimentation coefficient	$s^0_{20,w}$	$2.39 \cdot 10^{-13}$	$3.2 \cdot 10^{-13}$ $4 \cdot 10^{-13}$**	$3.5 \cdot 10^{-13}$
Diffusion coefficient	$D^0_{20,w}$	$7.9 \cdot 10^{-7}$	$9 \cdot 10^{-7}$ $9.5 \cdot 10^{-7}$**	
Partial specific volume	$\bar{v}$	0.742	0.749**	
Molecular weight				
Svedberg		29 000	31 100 40 000**	36 200
Yphantis		30 600		
Andrews		32 500		
Amino acid analysis		29 690 30 213 (approx.)		

* Ref. 11.
** Ref. 21.

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REFERENCES

1 M. L. ANSON, *J. Gen. Physiol.*, 22 (1938) 79.
2 K. ARIMA, S. IWASAKI AND G. TAMURA, *Annual Meeting Agr. Chem. Soc. Japan, 1962*, p. 51.
3 K. ARIMA, S. IWASAKI AND G. TAMURA, *Agr. Biol. Chem.*, 31 (1967) 540.
4 I. J. BABBAR, K. A. SRINIVASAN, S. C. CHAKRAVARTY AND A. T. DUADANI, *Indian J. Dairy Sci.*, 18 (1965) 89.
5 N. J. BERRIDGE, *Biochem. J.*, 39 (1945) 179.
6 DAVID A. YPHANTIS, *Ann. N.Y. Acad. Sci.*, 88 (1960) 586.
7 P. F. DYACHENCK AND V. V. SLAVAYANOVA, *Intern. Dairy Congr., 1962*, Sect. IV–1, 349.
8 I. EMUAUILOR, *Intern. Dairy Congr. Proc., 1956*, B401, 506.
9 JOSEPH L. SARDINAS, *Appl. Microbiol.*, 16 (1968) 248.
10 B. FOLTMAN AND S. HARTLEY, *Biochem. J.*, 104 (1967) 1064.
11 B. FOLTMAN, *Acta Chem. Scand.*, 17 (1963) 872.
12 T. W. GOODWIN AND R. A. MORTON, *Biochem. J.*, 4 (1946) 628.
13 S. IWASAKI, J. YU, G. TAMURA AND K. ARIMA, *Agr. Biol. Chem.*, 31 (1967) 546.
14 S. IWASAKI, J. YU, G. TAMURA AND K. ARIMA, *7th Abstr. Intern. Congr. Biochem. Tokyo, 1967*, IV, 758.
15 S. IRIE, Y. KANEZAWA AND T. IMAI, *Annual Meeting Agr. Chem. Soc. Japan, 1966*, p. 136.
16 S. G. KNIGHT, *Can. J. Microbiol.*, 12 (1966) 420.
17 S. MIZUSHIMA AND S. AKABORI, *Chem. Protein*, 2 (1954) 383, 385.
18 K. MORIHARA, *Agr. Chem. Soc. Japan*, 39 (1965) 514.
19 N. S. POLEVA AND N. V. POPOVA, *Ferment. Spirt. Prom.*, 31 (1965) 6.
20 Z. PUHAN, *Intern. Dairy Congr., 1966*, XVIID, 199.
21 H. SCHWANDER, P. ZAHLER and H. NITSCHMAN, *Helv. Chem. Acta*, 35 (1952) 553.
22 E. SCHRAM, S. MOORE AND E. J. BIGWOOD, *Biochem. J.*, 57 (1954) 33.
23 G. A. SOMUKUTI, F. J. BAVEL, *J. Dairy. Sci.*, 49 (1966) 700.
24 R. A. SRINIVASAN, *Appl. Microbiol.*, 12 (1964) 475.
25 T. SVEDBERG and K. O. PEDERSEN, *The Ultracentrifuge*, Clarendon, Oxford, 1940.
26 J. YU S. IWASAKI, G. TAMURA AND K. ARIMA, *Annual Meeting Agr. Chem. Soc. Japan, 1968*, p. 203.
27 J. YU, S. IWASAKI, G. TAMURA AND K. ARIMA, *Agr. Biol. Chem.*, 32 (1968) 1051.
28 K. ARIMA, J. YU, S. IWASAKI and G. TAMURA, *Appl. Microbiol.*, in the press.
29 B. FOLTMAN, *Acta Chem. Scand.*, 13 (1959) 1927.
30 J. YU, G. TAMURA AND K. ARIMA, *J. Agr. Chem. Soc. Japan*, in the press.
31 G. H. RICHARDSON AND J. H. NELSON, *J. Dairy Sci.*, 50 (1967) 1066.
32 S. IWASAKI, T. YASUI, G. TAMURA AND K. ARIMA, *Agr. Biol. Chem.*, 31 (1967) 1421.