Studies on Milk Clotting Enzymes Produced by Basidiomycetes

Part I. Screening Tests of Basidiomycetes for the Production of Milk Clotting Enzymes

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In the course of screening tests of Basidiomycete proteolytic enzymes, it was observed that some strains produced milk clotting enzymes with fairly weak proteolytic activities.

When sucrose-polypeptone and sucrose-corn steep liquor media were used, only 6 strains out of 44 strains tested showed weak milk clotting activities. Cheddar cheese making with culture filtrates of these 6 strains revealed that the culture filtrates of 2 strains, *Irpex lacteus* Fr. and *Fomitopsis pinicola* (Fr.) Karst., were able to produce Cheddar cheese of good quality.

On the other hand, when sucrose-distillers solubles media were used, a lot of strains showed high proteolytic activity in addition to high milk clotting activity. The ratio of milk clotting to proteolytic activities (MCA/PA) was assumed to be an important index for the selection of organism, and *F. pinicola* and *Coriolus consors* (Berk.) Imaz. were selected as the strain with high MCA/PA ratio.

As the investigation on culture conditions of 3 strains mentioned above showed that *F. pinicola* and *I. lacteus*, were richly productive of milk clotting enzymes, the 2 strains except *C. consors* were used for further studies on cheese making.

Cheddar cheese making with crude enzymes revealed that cheese products produced by the enzyme of *F. pinicola* had a slightly bitter taste after 5 months' ripening but that those produced by the enzyme of *I. lacteus* had good quality.

Because of the gradual shortage of the animal rennet prepared from the abomasums of young calves and the increasing demand for cheese products, the interest in rennet substitutes has been greatly increased in recent years.^{1,2)}

A great number of studies on rennet substitutes have been reported. According to Naudts,¹⁾ there are many milk clotting enzymes prepared from animals, vegetables^{3~8)}

and microbes.^{9~22)} The enzymes with a few exceptions thus far reported, however, had

1) Ir. M. Naudts, *International Dairy Federation*, Moscow, I-Doc 51 (1968).

2) H. A. Veringa, *Dairy Sci. Abst.*, **23**, 197 (1961).

3) R. Berkowitz-Hundert, J. Leibowitz and J. Ilany-Feigenbaum, *Enzymologia*, **27**, 332 (1964).

4) R. Berkowitz-Hundert, J. Ilany-Feigenbaum and J. Leibowitz, *Enzymologia*, **29**, 98 (1965).

5) R. Berkowitz-Hundert, *ibid.*, **31**, 281 (1966).

6) J. Ilany-Feigenbaum, R. Berkowitz-Hundert and J. Leibowitz, *ibid.*, **31**, 274 (1966).

7) M. A. Krishnaswamy, D. S. Johar, V. Subrahmanyam and S. P. Thomas, *Food Tech.*, **15**, 482 (1961).

8) K. Ramamurti and D. S. Johar, *Naturwiss.*, **51**, 88 (1964).

9) S. M. Barkan, O. Kb. Ramazanov and A. A. Yulius, *Dairy Sci. Abst.*, **27**, 152 (1965).

10) T. Kikuchi, *J. Food Sci. Tech.*, **15**, Abst. of 15th Meeting (1968).

11) S. G. Knight, *Can. J. Microbiol.*, **12**, 420 (1966).

12) N. P. Melachouris and S. L. Tuckey, *J. Dairy Sci.*, **50**, 943 (1967).

13) J. L. Sardinas, *Appl. Microbiol.*, **16**, 248 (1968).

high proteolytic activities in addition to milk clotting activities, so that the cheese produced by those enzymes had a soft curd texture or had an off-flavor, especially a bitter taste.

Only two enzymes, pepsin^{1,2)} and the enzyme produced from *Mucor pusillus*,¹⁵⁻²²⁾ have so far been regarded as the promising rennet substitutes.

In the course of studies on proteolytic enzymes from Basidiomycetes, it was observed that some strains produced milk clotting enzymes with fairly weak proteolytic activities. The present paper deals with the screening of Basidiomycetes for the production of milk clotting enzymes.

MATERIALS AND METHODS

Basidiomycete strains tested and the assay methods for proteolytic activity. Proteolytic activity (PA) was expressed in optical density at 660 m μ per one ml of culture filtrates. Assay techniques and the strains used were previously described.²³⁾

Compositions of culture media. A medium composed of 5% of sucrose, 0.3% of yeast extract, 0.1% of KH₂PO₄, and 0.05% of MgSO₄·7H₂O was used as a basal one, and to this medium 0.5% of polypeptone, 2% of corn steep liquor (CSL), or 3% of distillers solubles (SVP) was added as a nitrogen source.

Cultivating methods of Basidiomycete strains are

referred to the previous paper.²³⁾

An assay method for milk clotting activity. Twelve per cent solution of milk powder fortified with M/1000 CaCl₂·2H₂O was used as a substrate.

For assay of the enzyme activity 10 ml of the substrate solution, whose pH value was adjusted with 1N HCl exactly to 6.2, was added to 1 ml of enzyme solution of a suitable dilution at 35°C.

The time necessary for the formation of curd fragments was measured with a stop watch. Milk clotting activity (MCA) was defined as follows.

$$MCA = \frac{2400}{t(\text{sec})} \times \text{dilution coefficient}$$

t: Time necessary for the curd fragment formation.

Manufacture of Cheddar cheese.^{24,25)} To fresh milk pasteurized at 62°C for 30 min and cooled at 30°C 2% of starter was added, and immediately after titratable acidity of milk was adjusted to 0.2% as lactic acid by the addition of 1N HCl, enzymes or culture filtrates were added to the milk.

After 30 min, curd of milk was cut and gradually cooked from 30 to 37°C. Whey was removed when acidity of whey became 0.035% more than that measured immediately after the cutting. When acidity of whey draining from curd block during cheddaring became 0.45%, the curd was milled and blended with 2% of NaCl. After that the curd was hooped and pressed.

After 24 hr, the pressed cheese was coated with paraffin and stored in a refrigerator at 10~15°C.

RESULTS

1. Screening tests in the sucrose-polypeptone or the sucrose-CSL media

Results of screening tests when polypeptone or CSL was used as a nitrogen source are shown in Table I.

When organisms were cultivated in the sucrose-polypeptone medium, only 3 strains showed weak milk clotting activities, and in the sucrose-CSL medium 6 strains had also

14) R. A. Srinivasan, M. K. K. Iyengar, I. J. Babbar, S. C. Chakravorty, A. T. Dudani and K. K. Iya, *Appl. Microbiol.*, **12**, 475 (1964).

15) K. Arima, S. Iwasaki and G. Tamura, *Agr. Biol. Chem.*, **31**, 540 (1967).

16) S. Iwasaki, G. Tamura and K. Arima, *ibid.*, **31**, 546, 1421, 1427 (1967).

17) J. Yu, S. Iwasaki and K. Arima, *ibid.*, **32**, 1048, 1051 (1968).

18) G. A. Somkuti and F. J. Babel, *Appl. Microbiol.*, **15**, 1309 (1967).

19) G. A. Somkuti and F. J. Babel, *J. Bact.*, **95**, 1407, 1415 (1968).

20) G. H. Richardson, J. H. Nelson, R. E. Lubnow and R. L. Schwarbery, *J. Dairy Sci.*, **50**, 1066 (1967).

21) T. Tsugo, U. Yoshino, K. Taniguchi, A. Ozawa, A. Miki, S. Iwasaki and K. Arima, *Jap. J. Zootech. Sci.*, **35**, 221, 229 (1964).

22) M. E. Schulz, E. Voss, H. Sell und G. Mrowetz, *Milchwiss.*, **22**, 139 (1967).

23) M. Kawai and Y. Otsuka, *Trans. Mycol. Soc. Japan*, **10**(1), 29 (1969).

24) "Shokuhin Kako Ho," ed. by K. Osaki, Asakura Co., Tokyo, 1964, p. 411.

25) T. Yamamoto, K. Takahashi and M. Yoshino, *Bull. National Institute Agr. Sci.*, Series G, **15**, 69 (1958).

TABLE I. A RESULT OF SCREENING TESTS FOR THE PRODUCTION OF MILK CLOTTING ENZYMES IN THE SUCROSE-POLYPEPTONE AND THE SUCROSE-CSL MEDIUM

Nos. of strain	Species names	MCA	
		Polypeptone medium	CSL medium
1	<i>Coprinus macrorhizus f. microsporus</i>	0	0
2	" "	0	0
3	" "	0	0
4	<i>Coprinus micaceus</i>	0	0
5	" <i>radians</i>	0	0
6	" "	0	0
7	<i>Coprinus</i> sp.	0	0
8	<i>Naematoloma fasciculare</i>	0	0
9	<i>Oudemansiella radicata</i>	0	0
10	<i>Agaricus bisporus</i>	0	0
11	<i>Clavaria mucida</i>	0	0
12	<i>Flammulina velutipes</i>	0	0
13	<i>Lentinus edodes</i>	0	0
14	" "	0	0
15	<i>Pholiota nameko</i>	0	0
16	<i>Pleurotus ostreatus</i>	0	0
17	" "	0	0
18	<i>Armillaria mellea</i>	0	0
19	<i>Coriolus consors</i>	0	0
20	" "	0	0
21	" <i>hirsutus</i>	0	0.2
22	" <i>versicolor</i>	0	0.2
23	<i>Daedaleopsis styracina</i>	0.2	0.3
24	" "	0	0
25	<i>Elfvigia applanata</i>	0	0
26	<i>Fomes fomentarius</i>	0	0
27	<i>Fomitopsis annosa</i>	0	0
28	" <i>castanea</i>	0	0
29	" <i>insularis</i>	0	0
30	" "	0	0
31	" <i>officinalis</i>	0	0
32	" <i>pinicola</i>	0	0.5
33	" <i>cytisina</i>	0	0
34	<i>Fuscoporia obliqua</i>	0	0
35	<i>Irpex lacteus</i>	0.5	1.4
36	<i>Lampteromyces japonicus</i>	0	0
37	<i>Lenzites betulina</i>	0.5	0.5
38	<i>Gloeophyllum saepiarium</i>	0	0
39	" "	0	0
40	<i>Panellus stipticus</i>	0	0
41	<i>Phaeolus schweinitzii</i>	0	0
42	<i>Schizophyllum commune</i>	0	0
43	<i>Stereum hirsutus</i>	0	0
44	<i>Trametes sanguinea</i>	0	0

MCA: Milk clotting activity.

TABLE II. A RESULT OF SCREENING TESTS FOR THE PRODUCTION OF MILK CLOTTING ENZYMES IN THE SUCROSE-SVP MEDIUM

Nos. of strains	SVP medium (pH 4.5)			SVP medium (pH 5.5)		
	MCA	PA	MCA/PA	MCA	PA	MCA/PA
1	4.4	0.286	15.4	6.8	0.740	9.2
2	4.8	0.468	10.2			
3	2.9	0.441	6.6	7.3	1.340	5.4
4	0			0		
5	0			0		
6	0			0		
7	0			0		
8	0			0		
9	0			0		
10	0			0		
11	0			0		
12	2.1	0.522	4.0	2.2	0.885	2.5
13	0			0		
14	0			0		
15	0			0		
16	2.0	0.412	4.9	0		
17	1.4	0.595	2.4	2.0	0.365	5.5
18	0			1.0	0.150	6.7
19	1.5	0.145	10.3	13.0	0.950	13.7
20	4.5	0.079	57.0	3.3	0.025	132.0
21	4.4	0.310	14.0	25.3	1.055	24.0
22	13.7	0.655	20.9	7.8	0.285	27.4
23	12.6	0.655	19.2	15.5	1.150	13.5
24	0			0		
25	3.1	0.475	6.5	3.2	0.120	26.7
26	16.0	0.455	35.2	2.0	0.355	5.6
27	1.7	0.045	37.8	5.1	0.260	19.6
28	7.8	0.745	10.5	7.8	0.850	9.2
29	0			0		
30	0			0		
31	0			0		
32	7.8	0.165	47.2	10.9	0.415	26.3
33	4.6	1.830	2.5	7.8	1.515	5.1
34	0			0		
35	20.0	1.305	15.3	13.3	1.180	11.3
36	1.8	0.270	6.7	0		
37	12.6	0.705	17.9	6.4	1.010	6.3
38	4.4	0.335	13.1	2.0	0.350	5.7
39	0			0		
40	4.5	0.740	6.4	0		
41	0			0		
42	2.5	0.122	20.5	2.9	0.455	6.5
43	0			0		
44	43.6	1.545	28.2	16.0	1.240	12.9

MCA: Milk clotting activity, and PA: Proteolytic activity at pH 6.

weak activities, overlapping 3 strains mentioned above.

In both media the growth of strains tested was poor and mycelia of almost all strains formed large pellets. Probably because of poor growth in both media, only a few strains had detectable milk clotting activities.

Cheddar cheese making on a small laboratory scale²⁴ using the culture filtrates of those six strains revealed that normal cheese textures were made by the culture filtrates of *Fomitopsis pinicola* (Fr.) Karst. and *Irpex lacteus* Fr., but that soft curds were formed using those of other four strains.

After ripening of these cheese products at 10~15°C for 6 months, the ones produced by the culture filtrates of *F. pinicola* and *I. lacteus* had no off-flavor, but other products had an irritative bitter taste.

2. Screening tests in the sucrose-SVP medium

In the sucrose-SVP medium the growth of many strains was better than in the sucrose-polypeptone and the sucrose-CSL media, and almost all strains formed very small pellets or pulpy mycelia.

In this medium a lot of strains became to be able to clot milk (Table II), but at the same time proteolytic activities became also high.

As a rennet substitute it is necessary that the enzyme has not only high milk clotting activity, but also low proteolytic activity. Therefore, the ratios of milk clotting activity

to proteolytic activity (MCA/PA) of three rennets on the market, *i.e.* Hansen's calf rennet, *Mucor pusillus* rennet (Meito's rennet) and *Endothia parasitica* rennet (Pfizer's rennet), were measured and adopted as the index of screening tests (Table III).

As a result, two strains, *Coriolus consors* (Berk.) Imaz. and *F. pinicola*, were selected owing to their high MCA/PA ratio.

As the study on culture conditions of 3 strains mentioned above showed that *F. pinicola* and *I. lacteus* were richly productive of milk clotting enzymes the 2 strains except *C. consors* were used for further studies on cheese making.

Manufacture of Cheddar cheese revealed that cheese products produced by the enzyme of *F. pinicola* had a slightly bitter taste after 5 months' ripening but that those produced by the enzyme of *I. lacteus* had good quality.

DISCUSSION

Since almost all proteolytic enzymes clot milk, a great variety of proteolytic enzymes obtained from various sources were tested as animal rennet substitutes. Unfortunately, however, their strong proteolytic activity that caused curd peptonization or off-flavor, especially a bitter taste, prevented them from being used in cheese manufacture.

Attempts to separate milk clotting activity from proteolytic activity have so far been unsuccessful.¹⁴ Milk clotting activity seems to be invariably accompanied by proteolytic activity. This is why the ratio of milk clotting to proteolytic activity was referred to as the index for screening tests. If it is taken into consideration that in cheese manufacture pH values of fresh milk, when rennet preparations are added, is 6.2~6.3,²⁵ MCA/PA ratio at pH 6 will be the important index of organisms.

However, it is hard to decide, until the cheese products are actually produced and tasted, whether or not the products with a

TABLE III. SOME COMPARISONS OF CHARACTERISTICS BETWEEN A TRADITIONAL ANIMAL RENNET AND MICROBIAL RENNETS ON THE MARKET

		MCA/mg	PA/mg	MCA/PA
Animal rennet	Hansen's powder	15.0	0.090	167.0
	Pfizer's rennet	45.0	1.390	32.4
Microbial rennet	Meito's rennet	16.0	0.322	49.7

MCA: Milk clotting activity and PA: Proteolytic activity indicated in optical density at 660 m μ .

good flavor are able to be produced by the rennet substitutes in question.

Judging from these points, *I. lacteus*, by the enzyme of which good quality of Cheddar cheese could be produced, is chosen a promising strain.

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