Production and Some Properties of Rennin-like Milk-clotting Enzyme from *Penicillium citrinum*

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SUMMARY

The production of a rennin-like milk-clotting enzyme by *Penicillium citrinum* 805 was investigated. Corn-steep water was the best medium for the production of the enzyme. Dephytinization of corn-steep water lowered production of the enzyme. The extent of mycelial sporulation was correlated with the milk-clotting activity. Precipitation with ammonium sulphate and ethanol was unsuitable, but acetone produced active fractions. The enzyme action was optimal at 60° and at pH 6.0 while the stability of the enzyme was maximal at pH 5.37. The enzyme exhibited feeble proteolytic activity.

INTRODUCTION

All proteolytic enzymes can clot milk, but rennin is the main milk-clotting enzyme. A rennin enzyme or rennin substitute, suitable for cheese making, is characterized by a high milk-clotting activity and low proteolytic action. These enzymes occur in animal tissues and higher plants as well as in micro-organisms. Although much is known about enzymes of animal and plant origins, little is known about microbial milk-clotting enzymes. Attention has therefore recently focused on the production of milk-clotting enzymes from microbial sources for use as rennin substitutes (Srinivasan, Anantharamiah, Ananthakrishnan & Iya, 1962*a*; Srinivasan *et al.* 1964; Veselov, Tipograph & Petina, 1965; Iwasaki, Tamura & Arima, 1967; Osman, Abdel-Fattah, Abdel-Samie & Mabrouk, 1969; Paleva, 1969; Kawai, 1970; Kawai & Mukai, 1970). Successful attempts to use microbial milk-clotting enzymes have been made by some investigators (Srinivasan, Anantharamiah, Keshavamurthy, Ananthakrishnan & Iya, 1962*b*; Toshihiko & Shuji, 1969) and calcium phytate was reported by Srinivasan *et al.* (1964) to stimulate the production of milk-clotting enzymes of some *Bacillus* isolates and also of the proteases of some bacteria (Tsuychihiara, 1954).

The present work was undertaken to study the production of milk-clotting enzyme by *Penicillium citrinum* in different culture media including dephytinized corn-steep water, its partial purification, and some of its properties. These studies may throw some light on the value of the fungal enzyme preparation as a rennin-like enzyme.

METHODS

Organism. *Penicillium citrinum* 805 (NRRL) was used in this work.

Culture media. Thirteen media were used and are listed in Table 1.

Dephytinization of corn-steep water. This was done according to the method of Zelinka, Hudec & Pelcova (1961). A corn-steep water of 2 % (w/v) was used.

Cultivation. Transfers were made from the subcultures to Dox's agar plates which were then incubated at 30° for seven days. Cultivation was in 250 ml flat-bottomed flasks, each containing 50 ml of sterile medium. Four discs (0.5 cm diam.) were cut from the seven-day-old culture plates and used for inoculating each flask. After incubation for seven days at 30°, the culture medium was filtered and its volume adjusted to 50 ml.

Measurement of milk-clotting activity. This was done by the method of Berridge (1952); unless otherwise specified, 2.5 ml of the enzyme sample were incubated with 10 ml reconstituted skim-milk (12 g dry skim-milk/100 ml 0.01 M-CaCl₂) at 40° and the clotting time recorded. One unit of enzyme activity was taken to be that which clotted 10 ml milk in 10 min.

Estimation of protein content. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951).

Examination of gelatinase activity. This was done according to the method of Bergkvist (1963) for proteases of *Aspergillus oryzae* by estimating the decrease in the relative viscosity of a 5% (w/v) buffered gelatin solution (citrate + phosphate buffers of different pH values) containing the enzyme.

Examination of caseinase activity. This was done according to the method of Bergkvist (1963) by determining the rate of hydrolysis of 1.5% (w/v) casein (isoelectric casein) in phosphate buffer (pH 7.4) containing the enzyme, after incubation for 30 min at 37°. The increase in extinction (280 nm) of trichloroacetic acid filtrate was used as a measure of proteolytic activity.

Precipitation of milk-clotting enzyme. This was achieved with ammonium sulphate, ethanol or acetone using the culture filtrate obtained by culturing the organism on the corn-steep water (2%, w/v) + lactose (2%, w/v) medium. With ammonium sulphate, centrifugation was at 11,000 rev./min and with ethanol or acetone at 3000 rev./min. Suspensions of ammonium sulphate precipitates and their supernatant fluids were dialysed against distilled water while each of the other precipitates was resuspended in a known volume of water.

Properties of milk-clotting enzyme. These were studied in the precipitate obtained by treating the culture filtrate with 2 vol. ice-cold acetone.

RESULTS AND DISCUSSION

Production of milk-clotting enzyme. Corn-steep water proved to be the best medium for the production of extracellular milk-clotting enzyme (Table 1). Considerable activity was also achieved by supplementing corn-steep water with lactose. Dephytinization of corn-steep water resulted in almost complete absence of milk-clotting activity. This may have been partially due to the removal of phytic acid (or related compound) from the corn-steep water which is known to contain this compound (Graefe, 1952; Abdel-Akher & Michalinos, 1962). The recovery of milk-clotting activity by the addition of inositol + potassium dihydrogen phosphate to the dephytinized corn-steep water may support this view. This result is in agreement with that of Srinivasan *et al.* (1964) who found that calcium phytate stimulated the production of milk-clotting enzyme in bacteria. The addition of either inositol or casaminoacids had an adverse effect on the production of milk-clotting enzyme. Skim-milk was unsuitable for its production.

When the culture filtrate showed weak or no milk-clotting activity, the corresponding mycelia were partially or completely non-sporing. The association of milk-clotting activity with spore formation was also observed by Osman *et al.* (1969).

Partial purification of the milk-clotting enzyme. Ammonium sulphate was unsuitable for

salting out active fractions of the milk-clotting enzyme (Table 2). Although all precipitates possessed weak activities, two were more active. These had been salted out with ammonium sulphate of 30 and 100 % saturations. Their activities represented about 27 and 16.88 %, respectively, of that of the culture filtrate.

Ethyl alcohol was also unsuitable and gave precipitates with no activity with the exception of two which were obtained by treating the culture filtrate with 2 and 8 vol. of alcohol (Table 2). However, both possessed only weak milk-clotting activity.

Acetone gave the most active precipitates, and 2 vol. yielded a precipitate with even higher milk-clotting activity than the culture filtrate (Table 2). Gradual loss of enzyme activity occurred on precipitation with higher acetone concentrations.

Properties of the milk-clotting enzyme. The milk-clotting activity of the enzyme was maximal at 60° but nearly identical activity was achieved at 55°. This result is similar to those found for other microbial milk-clotting enzymes (Osman *et al.* 1969; Kawai & Mukai, 1970; Arnold, Stefan & Wanda, 1970). The enzyme showed maximal milk-clotting activity at

Table 1. *Production of milk-clotting enzyme by Penicillium citrinum 805 in different culture media*

Medium	Milk-clotting activity (units/mg protein)
CSW (2 %, w/v)	0.3172
CSW (2 %, w/v) + lactose (2 %, w/v)	0.2670
CSW (2 %, w/v) + glucose (2 %, w/v)	0.2380
CSW (2 %, w/v) + lactose (2 %, w/v) + inositol (0.1 %, w/v)	0.2380
CSW (2 %, w/v) + lactose (2 %, w/v) + inositol (0.2 %, w/v)	0.1070
CSW (2 %, w/v) + lactose (2 %, w/v) + KH_2PO_4 (0.1 %, w/v)	0.2480
CSW (2 %, w/v) + lactose (2 %, w/v) + casaminoacids (0.1 %, w/v)	0.1490
Dephytinized CSW	0.0019
Dephytinized CSW + lactose (2 %, w/v)	NC
Dephytinized CSW + lactose (2 %, w/v) + inositol (0.1 %, w/v) + KH_2PO_4 (0.1 %, w/v)	0.1728
Skim-milk solution (10 %, w/v)	NC
Skim-milk solution (10 %, w/v) + glucose (1 %, w/v)	0.0011
Skim-milk solution (10 %, w/v) + glucose (1 %, w/v) + CaCl_2 (0.005 M)	0.0012

CSW = corn-steep water; NC = no clotting. See Methods for definition of unit of milk-clotting activity.

Table 2. *Milk-clotting activity of ammonium sulphate, ethanol and acetone precipitates of Penicillium citrinum enzyme preparations*

Milk-clotting activity (unit/mg protein) (1) at various ammonium sulphate saturations (%) and (2) in various proportions (v/v) of culture filtrate + acetone (a) and ethanol (e).

(1)	30	50	66	80	100
ppt	0.173	0.084	0.079	0.082	0.108
supt	0.168	0.045	0.072	0.103	0.083
(2)	1 + 1	1 + 2	1 + 4	1 + 6	1 + 8
ppt (a)	NC	0.67	0.43	0.24	0.24
ppt (e)	NC	0.24	NC	NC	0.05

Activity of original culture filtrate was 0.64 unit/mg protein. ppt = precipitate; supt = supernatant; NC = no clotting.

pH 6.0; 1.45 mg enzyme protein in 2.5 ml was sufficient to clot 1.2 g reconstituted skim-milk in 10 ml within 148 s at pH 6.0 and 60°. Thus the fungal enzyme quickly clotted about 827 times its own weight of skim milk. In this respect, the milk-clotting enzyme preparation of *Penicillium citrinum* was superior to the bacterial rennin which was reported by Srinivasan, Anantharamiah & Lakashmi (1959) to clot the same amount of milk within 20 min.

A substrate concentration (reconstituted skim-milk) higher than 12 g/100 ml was a limiting factor for milk-clotting activity. The addition of proportional amounts of calcium chloride to skim-milk suspensions enhanced the clotting stage of enzyme action but the activity was not completely recovered, perhaps because of the resulting unfavourable reaction medium or the high substrate/enzyme ratio or both.

Although the milk-clotting activity was maximal at 60° (pH 6.0), it was destroyed when heated for 10 min at 55° and pH 3.42 in the absence of substrate. At 40° the milk-clotting enzyme was most stable at pH 5.37 (Table 3).

The milk-clotting enzyme had a low proteolytic activity. After incubation with casein, the increase in optical density of the trichloroacetic acid filtrate was only 0.018, indicating low proteolytic action (Bergkvist, 1963). Also, the enzyme acted feebly on gelatin solutions as compared with fungal proteolytic enzymes (Bergkvist, 1963). In a reaction lasting for 5 min the maximum reduction in relative viscosity of gelatin solution, pH 4.0, was 5.2%. At lower and higher pH values, reduction in viscosity did not exceed 3.4%. When the time of reaction at pH 4.0 was extended to 90 min, there was still only 17.4% reduction in viscosity.

The results show that the milk-clotting enzyme preparation of *Penicillium citrinum* possessed high milk-clotting activity and low proteolytic action, and accordingly may be considered as a rennin-like enzyme.

Table 3. *The pH stability at 40° of the partially purified milk-clotting enzyme preparation from Penicillium citrinum*

pH of enzyme solution	Milk-clotting activity (unit/mg protein) at different times of incubation of enzyme solution* (min)				
	10	30	60	120	180
3.42	6.27	5.25	4.28	2.85	1.50
4.05	6.20	5.10	4.80	3.61	3.06
4.63	6.69	6.44	6.29	5.47	4.69
4.99	7.38	7.06	6.46	5.71	5.15
5.37	8.36	7.67	6.51	5.84	4.93
5.89	7.74	7.71	6.83	4.61	2.63

* Before incubation with milk, the pH of the enzyme solution was readjusted to 4.05 (acetate buffer, 0.02 M).

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