Production of a Milk-clotting Enzyme Preparation by *Aspergillus niger* and the Effect of Various Factors on its Activity

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SUMMARY

Among 20 locally isolated fungi, *Aspergillus niger* isolate no. 58 proved to be suitable for the production of active extracellular milk-clotting enzyme. The addition of acetate buffer to the reaction mixture enhanced the clotting of milk by the enzyme whereas citrate + phosphate buffer hindered the process. The general properties of the crude enzyme were studied. Precipitation with ammonium sulphate, ethanol, acetone and tannin showed that ammonium sulphate was unsuitable for precipitation while the other precipitants produced sufficiently active fractions.

INTRODUCTION

Studies on the production of milk-clotting enzymes from microbial sources have gained interest during the last decade; the production of milk-clotting enzymes by different types of micro-organisms has been reported (Arima & Iwasaki, 1964; Veselov, Tipograf & Petina, 1965; Knight, 1966). The increasing demands on rennin for cheese making in Egypt and the prohibition of the slaughter of calves justified a study aiming to search for rennin substitutes from sources other than animals and in particular from micro-organisms.

METHODS

Micro-organisms. The fungi used in the present work were taken from the fungal collection of the Centre of Cultures of the National Research Centre, Cairo.

Culture media. The following media were used. Medium 1: Corn-steep liquor (2 %, w/v) + lactose (1 %, w/v) in water; Medium 2: Cane black-strap molasses (12 %, w/v) in water; Medium 3: Cane black-strap molasses (12 %, w/v) + lactose (1 %, w/v) in water; Medium 4: Milk whey alone; Medium 5: Lactose (2 %, w/v) in milk whey; Medium 6: A defined medium prepared according to Prill, Wenck & Peterson (1935) containing (g./l.): glucose, 50; NH_4NO_3 , 10; KH_2PO_4 , 6.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.16; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05.

Cultivation. Transfers were made from the stock slants to Dox agar plates which were then incubated at 30° for 7 days. Two discs (0.5 cm. diam.) were cut from the 7-day culture and inoculated into 50 ml. sterile medium in 250 ml. flat bottomed flasks. Unless otherwise specified, the flasks were incubated for 3 to 4 days at 30°. At the end of the incubation period the culture medium was filtered off and its volume adjusted to 50 ml.

Measurement of milk-clotting activity. The method of Berridge (1952) was used; 2.5 ml. of the enzyme sample were incubated with 10 ml. reconstituted skim milk (12 g. dry skim milk/100 ml. of 0.01 M-CaCl₂) at 40° and the clotting time recorded. In the present work, one unit is considered to be the amount of the enzyme in 2.5 ml. enzyme preparation which clots 10 ml. milk in 10 min. at 40°.

Proteolytic activity. The proteolytic activity of the enzyme sample was measured on the basis of the gelatinase test of Bergkvist (1963), who used it for determining the proteolytic activity of proteases produced by *Aspergillus oryzae*. The gelatinase activity was estimated as the decrease in the relative viscosity of a 5% (w/v) buffered gelatin solution containing the enzyme (acetate buffer of different pH values).

Precipitation of milk-clotting enzyme. This was achieved with ammonium sulphate, ethanol, acetone or tannin. With ammonium sulphate, the dialysed solutions of the precipitates and supernatant fluids were adjusted to pH 4.0 with acetate buffer. Precipitation with tannin was according to the method of Bergkvist (1963) and the precipitates freed from tannin by repeated dispersal in acetone during centrifugation. Each precipitate was resuspended in 0.02 M-acetate buffer (pH 4.0) and made to a known value with water.

RESULTS

Examination of the fungal cultures for production of milk-clotting enzyme. Medium 1 was used for cultivation. Of the 20 fungal cultures tested, surface cultures of *Aspergillus niger* no. 58 exhibited the highest milk-clotting activity (Table 1). The activity was always associated with spore formation. Thus, non-sporulating cultures, no matter how old, did not exhibit milk-clotting activity. *Aspergillus niger* no. 58 was, therefore, used in the succeeding experiments.

Table 1. Survey of fungal cultures for the production of milk-clotting enzyme

Time of incubation (days)..... Micro-organisms	Surface culture			Shaken culture		
	3	7	10	3	7	10
	Clotting-time of milk (min.)					
<i>Aspergillus fischeri</i> 108	-ve	-ve	240	240	240	300
<i>A. flavus</i> 112	75	120	240	45	55	180
<i>A. fumigatus</i> 109	90	180	-ve	105	180	-ve
<i>A. niger</i> 3N	-ve	35	120	55	30	80
<i>A. niger</i> 58	15	90	-ve	105	105	-ve
<i>Cunninghamella</i> sp. 114	-ve	300	240	-ve	270	270
<i>Epicoccum purpurosence</i> 5	-ve	40	30	-ve	30	30
<i>Fusarium equiseti</i> 8	-ve	165	-ve	-ve	-ve	-ve
<i>F. oxysporium</i> 6	-ve	195	300	-ve	360	240
<i>F. semitectum</i>	-ve	45	-ve	-ve	-ve	-ve
<i>F. sambucinum</i> 7	-ve	-ve	-ve	-ve	-ve	-ve
<i>Geotrichum candidum</i>	-ve	300	360	-ve	-ve	-ve
<i>Mucor</i> sp. 101	-ve	300	180	-ve	-ve	-ve
<i>Oospora</i> sp. 3	-ve	-ve	-ve	-ve	-ve	-ve
<i>Penicillium chrysogenum</i> 102	-ve	90	300	-ve	180	300
<i>P. notatum</i> 62	-ve	-ve	300	-ve	-ve	-ve
<i>P. notatum</i> 105	-ve	-ve	-ve	-ve	-ve	-ve
<i>P. ochraceus</i> Wilhelm 67	-ve	60	180	-ve	90	-ve
<i>Stemphylium consortiale</i> 21	-ve	210	180	-ve	210	240
<i>Trichoderma viride</i> 60	-ve	-ve	300	330	360	360

-ve: No clotting.

Suitability of different media for the production of milk-clotting enzyme by Aspergillus niger no. 58. The activity of the milk-clotting enzyme depended on the composition of the medium (Table 2). Undiluted whey medium fulfilled all the requirements for maximum production of enzyme activity. This result was of importance because of the possibility of conducting enzyme production and cheese production in one place. The immediate precipitation in the clotting test brought about by the medium 3 culture filtrate did not denote the presence of milk-clotting enzyme but was probably due to the acidity of the reaction mixture.

Table 2. *Production of milk-clotting enzyme by Aspergillus niger* 58 in different culture media

Medium	Final pH value of culture filtrate	Clotting-time of milk (min.)
Corn steep liquor (2 %, w/v) + lactose (1 %, w/v)	3.7	10
Whey	2.5	10
Whey + lactose (2 %, w/v)	3.0	40
Molasses (12 %, w/v)	4.0	60
Molasses (12 %, w/v) + lactose (1 %, w/v)	2.5	Immediate precipitation (no clotting)

Table 3. *Effect of buffers on milk-clotting activity of Aspergillus* enzyme

Buffer	pH value of buffer	pH value of reaction mixture	Clotting-time of reaction mixture (min.)
Acetate	5.0	6.0	1.92
	5.2	6.0	3.08
	5.4	6.2	3.66
	5.6	6.3	10.83
	5.9	6.4	18.75
Clotting-time of control: 45 min.			
McIlvaine	5.2	6.0	75.00
	5.4	6.0	75.00
	5.6	6.0	no clotting
	5.8	6.1	no clotting
Clotting-time of control: 25 min.			

In spite of the low pH values and the formation of good mycelial growth obtained with *Aspergillus niger* no. 58 grown on the defined medium no. 6, no milk-clotting activity was detected in the culture filtrate. Cultures which did not produce spores did not show any milk-clotting activity in the culture filtrates. Some relationship seems to exist between the formation of spores and the production of milk-clotting enzymes.

Effect of buffers on milk-clotting activity of Aspergillus niger no. 58 culture filtrates. In this experiment and the succeeding ones medium 1 was used for growth. The buffers tested were acetate and McIlvaine citrate + phosphate buffers at different pH values. The clotting of milk by the *Aspergillus* enzyme was accelerated by calcium ions, and retarded by citrate + phosphate, presumably owing to the combination with calcium (Table 3).

The influence of various factors on milk-clotting activity of Aspergillus niger no. 58 culture filtrate. The age of the culture was found to influence markedly the milk-

clotting activity. The 3-day culture filtrate of *A. niger* no. 58 gave the highest activity. The mycelial mats were completely sporulated at that stage of growth. After the third day of incubation, gradual loss of activity occurred accompanied by an increase in pH value of the culture.

As regards the effect of enzyme concentration, it was found that the smaller amounts of enzyme were being destroyed in the milk and that the larger amounts were to some extent inhibited, possibly as a result of slowness in the second phase of clotting.

The milk-clotting activity was optimal at 52°; such a high optimal temperature is normal for microbial milk-clotting enzymes (Tsugo *et al.* 1964). The effect of heat on the crude enzyme preparation indicated that the enzyme was thermolabile; it was inactivated when heated at 60° for 30 min. This result is in accordance with that obtained by other investigators for a microbial clotting enzyme which is thus relatively more heat resistant than are commercial rennet preparations (Tsugo *et al.* 1964). At 53° the crude enzyme retained its full activity when heated for 15 min. although, at the same temperature, a loss of activity of about 20 % occurred when the enzyme was left to react with milk without preheating. This latter phenomenon may be attributed to the loss of CO₂ from the milk, causing the pH value to increase.

Table 4. *Proteolytic (gelatinase) activity of the crude milk-clotting enzyme from Aspergillus niger 58 culture filtrate*

pH value of reaction mixture	Digestion periods (min.)						
	5.0	15	30	45	60	90	120
	% Decrease in relative viscosity						
3.40	17.00	22.03	26.00	26.00	29.37	34.00	35.59
4.05	17.51	24.29	27.11	29.37	29.94	34.00	35.59
4.99	18.64	23.16	25.42	27.11	29.37	29.94	34.46
5.89	21.46	26.00	27.11	29.37	29.37	29.94	32.20

Table 5. *The milk-clotting activity of the ethanol precipitates of Aspergillus enzyme preparations*

Culture filtrate + ethanol (v/v)	1 + 1	1 + 2	1 + 4	1 + 6	1 + 8
Activity relative to culture filtrate (units/2.5 ml.)	0.17	1.41	1.22	1.30	0.90

Table 6. *The milk-clotting activity of acetone precipitates of Aspergillus enzyme preparations*

Culture filtrate + acetone (v/v)	1 + 1	1 + 2	1 + 4	1 + 6	1 + 8
Activity relative to culture filtrate (units/2.5 ml.)	0.15	1.60	1.20	1.10	0.96

The gelatinase activity of the culture filtrate indicated a weak proteolytic action (Table 4) as compared with the results presented by Bergkvist (1963) for the proteases of *Aspergillus oryzae*. The latter author found a significant decrease in the viscosity of gelatin, reaching about 70 % after digestion for only 5 min.

The partial purification of the microbial milk-clotting enzyme by precipitation with some agents indicated that ammonium sulphate was unsuitable since a marked loss of enzyme activity occurred as soon as enough salt to cause precipitation was added.

Precipitation with ethanol or acetone gave precipitates with good milk-clotting activity; two volumes of either solvent gave the most active precipitates (Tables 5, 6). These results are more or less in accordance with those found by Iwasaki, Tamura & Arima (1967).

Table 7. *The effect of tannin concentration on the milk-clotting activity of the precipitates of Aspergillus enzyme preparations from culture filtrate of pH 5.5*

Tannin concentration (% w/v)	0.1	0.3	0.5	0.7	0.9	1.0
Activity relative to culture filtrate (units/2.5 ml.)	0.02	0.37	0.71	0.83	1.4	0.65

Table 8. *The effect of the pH value of the culture filtrate on the milk-clotting activity of precipitates by 0.9% (w/v) tannin*

pH value of culture filtrate	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Activity relative to culture filtrate (units/2.5 ml.)	1.22	1.29	1.30	1.40	0.88	0.67	0.0

Precipitation with tannin gave active precipitates. The extent of the recovered activity depended on tannin concentration (Table 7) as well as the pH value of the culture filtrate (Table 8). These results are in accordance with those found by Bergkvist (1963) for the proteolytic enzymes of *Aspergillus oryzae*.

This paper is dedicated to the memory of the late Professor E. M. Taha, who suggested the topic of this work.

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