Studies on the Production of Milk-clotting Enzymes, Proteolytic Enzymes and Mucilage by Fungi

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(Received 12 November 1973; revised 30 April 1974)

SUMMARY

Of 48 fungal isolates, 28 (including one *Penicillium* and three *Aspergilli*) secreted milk-clotting enzymes. This activity was in general related to the mycelial growth of a given isolate, but not with pH change of the culture filtrate during incubation. It was not found when mucilage was formed by isolates capable of doing so. The ratio of general proteolytic activity to milk-clotting activity varied from one organism to another, and with the age of culture of the same fungal strain.

INTRODUCTION

Attention has recently been focused on milk-clotting enzymes from micro-organisms for use as rennin substitutes (Veringa, 1961; Davis, 1970) and various fungi have been investigated for this purpose (Veselov, Tipograph & Petina, 1965; Arima, Iwasaki & Tamura, 1967; Paleva, 1969; Abdel-Fattah, Mabrouk & El-Hawwary, 1972*a, b*). The association of general proteolytic activity with most milk-clotting enzymes, however, has led to a search for micro-organisms producing the latter with low general proteolytic action (Arima *et al.* 1967; Abdel-Fattah *et al.* 1972*a*). In the present work, various fungi were screened for the production of these activities. A hitherto unreported phenomenon relating the production by some fungi of milk-clotting enzymes and mucilage was also examined.

METHODS

Organisms. The fungal strains used in the present work were obtained from the United States Department of Agriculture, Agricultural Research Service, Northern Utilization Research and Development Division, Peoria, Illinois 61604, U.S.A. (NRRL) and from the fungal collection of the Centre of Cultures of the National Research Centre, Cairo, Egypt. *Pseudoarachnietus roseus* (*Arachnietus dankallensis*), a local isolate, was kindly provided by Professor H. M. Youssef, Botany Department, Faculty of Science, Alexandria University, Egypt.

Culture. Unless otherwise specified, the medium used was corn-steep solids (2 %, w/v) plus lactose (2 %, w/v). Transfers were made from the subcultures to Dox's agar plates, which were then incubated at 30 °C for 7 days. Liquid cultures were grown in 250 ml flat-bottomed flasks, each containing 50 ml sterile medium. Four discs, each of 5 mm diam, were cut from the 7-day-old culture plates and used for inoculating each flask. After incubation for different

Table 1. *Milk-clotting activity of fungal cultures*

Type of culture: surface, 7 day incubation.

Micro-organism	pH of culture filtrate	Protein content of culture filtrate (mg/2.5 ml)	Clotting time* (min)
<i>Aspergillus fischeri</i>	4.2	2.13	58
<i>A. flavus</i>	3.8	4.13	50
<i>A. giganteus</i>	3.7	5.25	70
<i>A. hennenbergii</i> NRRL1736	6.8	5.12	270
<i>A. niger</i> 46H	4.7	6.63	360
<i>A. niger</i> NRRL372	6.7	5.25	63
<i>A. niger</i> NRRL393	6.1	5.87	255
<i>A. niger</i> NRRL604	6.0	5.75	20
<i>A. niger</i> NRRL611	6.2	7.50	24
<i>A. niger</i> NRRL615	5.6	5.62	17
<i>A. versicolor</i>	3.0	3.62	20
<i>Cladosporium</i> sp. 56	2.8	22.20	5
<i>Cunninghamella echinulata</i>	2.8	19.00	2
<i>Epicoccum purpurascens</i>	6.2	7.50	240
<i>Mucor hiemalis</i>	5.9	4.75	75
<i>Penicillium chrysogenum</i>	2.8	22.00	1.5
<i>P. expansum</i>	5.3	4.12	13
<i>P. martensii</i>	2.5	23.20	4

* Filtrates of these isolates produced clotting after overnight incubation (12 h): *Aspergillus niger* NRRL335, *Curvularia lunata*, *Fusarium moniliforme*, *F. semitectum* 9, *Gibberella* sp. 117, *Mucor fragilis*, *Oospora* sp. 3, *Penicillium notatum* 61, *Rhizopus nigricans*, *Trichoderma viride*. Filtrates of these isolates produced no clotting after 12 h: *Alternaria tenuis*, *Aspergillus clavatus*, *A. funiculosus*, *A. niger* 66, *A. niger* 73, *Chaetomium globosum*, *Cladosporium* sp. 54, *Cunninghamella* sp. 228, *Fusarium equiseti*, *F. oxysporum*, *F. semitectum* 4, *Geotrichum candidum*, *Helminthosporium spiciferum* (*Cochliobolus spicifer*), *Penicillium griseofulvum* NRRL2300, *P. lilacinum*, *P. notatum* 62, *Stemphylium* (*Ulocladium*) *consortiale*, *Trichothecium roseum*, *Verticillium dahliae*. Under similar conditions, 2 mg calf rennin (Difco) in 2.5 ml water, clotted reconstituted skim milk (10 ml) in 7 min.

periods at 30 °C, the culture medium from each flask was filtered off and made up to 50 ml with distilled water.

Protein determination. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951).

Assay of milk-clotting activity. This was done according to the method of Berridge (1952); 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 °C and the clotting time recorded. One unit of enzyme activity was taken to be that which clotted 10 ml milk in 10 min.

Assay of general proteolytic activity. This was done according to the method of Bergkvist (1963) by determining the rate of hydrolysis of 1.5 % (w/v) casein in 0.2 M-sodium phosphate buffer (pH 7.4) containing the enzyme, after incubation for 30 min at 37 °C. The increase in E_{280} of trichloroacetic acid filtrate was used as a measure of proteolytic activity; the values were calculated on the basis of mg enzyme protein.

RESULTS AND DISCUSSION

Of the 48 fungal isolates investigated only 28 brought about clotting of milk (Table 1). On the other hand, of 16 *Aspergillus* strains 12 produced milk-clotting enzymes of differing activities. *Aspergillus niger* 604, *A. niger* 615 and, especially, *A. versicolor*, were the more potent *Aspergillus* strains for producing active milk-clotting enzymes.

Table 2. Milk-clotting activity, proteolytic activity and their ratio in culture filtrates of some fungal isolates

Type of culture: surface.							
Micro-organism	Time of incubation (days)	pH of culture filtrates	Protein content of culture filtrates (mg/2.5 ml)	Dry wt of mycelium (mg/50 ml medium)	Milk-clotting activity, MC, (unit/mg protein)	Proteolytic activity, P*	MC/P†
<i>Aspergillus fischeria</i>	4	3.3	5.50	351	0.03	0.00	(∞)
	7	4.2	2.13	625	0.08	0.00	(∞)
<i>A. flavus</i>	4	4.0	6.00	763	0.02	0.02	1.00
	7	3.8	4.13	1032	0.05	0.00	(∞)
<i>A. giganteus</i>	7‡	3.7	5.25	WG	0.03	0.00	(∞)
<i>A. hennenbergii</i> NRRL 736	4	3.3	4.50	972	0.04	0.00	(∞)
	7	6.8	5.12	917	0.01	0.06	0.12
<i>A. niger</i> NRRL 372	4	3.6	6.00	1033	0.06	0.00	(∞)
	7	6.7	5.25	846	0.03	0.01	2.00
<i>A. niger</i> NRRL 393	4	4.1	4.50	932	0.01	0.02	0.38
	7	6.1	5.87	854	0.01	0.00	(∞)
<i>A. niger</i> NRRL 604	4	3.5	5.37	978	0.11	0.00	(∞)
	7	6.0	5.75	780	0.09	0.00	(∞)
<i>A. niger</i> NRRL 611	4	4.1	5.37	926	0.11	0.00	(∞)
	7	6.2	7.50	822	0.06	0.01	4.00
<i>A. niger</i> NRRL 615	4	3.6	4.25	919	0.09	0.01	6.71
	7	5.6	5.62	1155	0.10	0.02	5.53
<i>A. versicolor</i>	4	3.5	4.75	568	0.09	0.02	4.63
	7	3.0	3.62	598	0.14	0.00	(∞)
<i>Mucor hiemalis</i>	4	5.6	6.12	204	0.02	0.00	(∞)
	7	5.9	4.75	323	0.03	0.07	0.39
<i>Epicoecum purpurascens</i>	4	3.6	6.12	957	0.01	0.04	0.22
	7	6.2	7.50	887	0.01	0.00	(∞)
<i>Penicillium expansum</i>	4	3.4	7.87	WG	0.07	0.00	(∞)
	7	5.3	4.12	WG	0.19	0.00	(∞)

WG, Weak growth.

* As ΔE_{280} of the trichloroacetic acid filtrate after incubation with casein.

† Calculated before 'rounding off'.

‡ No growth at 4 days.

Variations in the production of milk-clotting enzymes by *Penicillium* strains were also noted. Thus among the seven *Penicillium* strains tested, the culture filtrates of *P. expansum*, *P. notatum* 61, *P. chrysogenum* and *P. martensii* clotted milk. Although the two last-named *Penicillium* strains produced the most active milk-clotting enzymes, the filtrates possessed high general proteolytic activity because of peptonization of the curds formed. The milk-clotting action brought about by the culture filtrates of *P. chrysogenum* and *P. martensii* was therefore presumed to be due to general proteolytic action rather than to milk-clotting enzymes with limited proteolytic action. The same was also observed with culture filtrates of *Cladosporium* sp. 56 and *Cunninghamella echinulata*. The culture filtrates of other strains of these genera either possessed feeble milk-clotting activity, or none.

Curvularia lunata, *Fusarium moniliforme* and *Pseudoarachniotus roseus* produced mucilaginous materials coating the mycelia, but the culture filtrates possessed very weak milk-clotting activities. This phenomenon was further investigated by growing these isolates for

4 and 7 days (surface and shaken cultures) on a medium composed of (% w/v): corn-steep solids (2), asparagine (0.7), glucose (2) and peptone (1.5). Results were similar, and mycelial growth was good. Abdel-Fattah *et al.* (1972*b*) reported that *Penicillium citrinum* produced active milk-clotting enzyme when cultured on a corn steep plus lactose medium. However, when the same isolate was cultured on skim milk solution, the culture filtrate possessed no milk-clotting activity and the mycelium was associated with mucilaginous material (Abdel-Fattah *et al.* 1972*b*). An inverse relationship may thus exist between the production of milk-clotting enzymes and the formation of mucilage by some fungi.

As shown in Table 1, no relationship seemed to exist between the pH of a culture filtrate and its milk-clotting activity. Thus, though, in some isolates, culture filtrates of low pH (3.0) quickly clotted milk, others of the same pH showed no activity. The pH values of all the reaction mixtures (culture filtrate + milk) varied only within a limited range, from 6.0 to 6.3, probably because of the high buffering capacity of milk. The extent of milk-clotting activity was also independent of the total protein content of the culture filtrate.

To select a rennin-like milk-clotting enzyme, i.e. one with limited general proteolytic action, the tests on fungi were repeated with strains which showed milk-clotting activity without peptonization of curd (hence excluding *Cladosporium* sp. 56, *P. chrysogenum*, *P. martensii* and *Cunninghamella echinulata*). In this investigation each fungus was cultured for 4 and 7 days and the milk-clotting activity, proteolytic activity and the ratio between these activities were determined.

The data recorded in Table 2 indicate that the incubation time influenced the milk-clotting activity of the culture filtrate. With most of the fungal cultures investigated, this activity was related to mycelial growth, but not to pH changes of the culture filtrate during incubations. No consistent relationships were found between general proteolytic activity and the pH, the protein content of the culture filtrate, or mycelial growth.

The ratio of milk-clotting activity to general proteolytic activity for an enzyme preparation is an index of its suitability as a rennin substitute. According to the results, *Aspergillus fischeri*, *A. giganteus*, *A. niger* 604 and, especially, *Penicillium expansum* appeared the most promising fungal isolates for production of rennin-like enzymes.

The results showed that milk-clotting and general proteolytic activities are different. Thus where association of the two enzymic activities occurred, their ratio varied not only from one isolate to another but also differed for the same fungal strain with the age of culture. These results are in agreement with those previously reported (Srinivasan *et al.* 1964; Abdel-Fattah *et al.* 1972*a*).

The authors thank Professor H. M. Youssef, Botany Department, Faculty of Science, Alexandria University, Egypt, for his kind donation of the mucilage-producing fungal culture *Pseudoarachniotus roseus* and for his interest throughout the work.

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