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PRODUCTION AND SOME IMPORTANT PROPERTIES OF A PARTIALLY PURIFIED RENNIN-LIKE EXTRACELLULAR ENZYME FROM *FUSARIUM SUBGLUTINANS* (WOLLENWEBER & REINKING) NELSON *et al* GROWN STATICALLY

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Production of rennin-like extracellular enzyme was studied in static cultures of *Fusarium subglutinans.* Maximum production (648.5 SU/ml) was obtained at 30°C, pH value of 4.5, after 7 days of incubation where the fermentation medium was composed of 2% ,w/v, wheat flour. Production of this enzyme is assumed to be growth-associated type. Iso-propanol at the ratio of 1:1 (v/v) among different precipitating agents was selectively used to obtain partially purified enzyme that retains 28.37% of its original activity with 2.28-fold purification. Such partially purified enzyme was found active maximally at 55°C and pH value of 6.0 and was stable below 55°C for 60 min and in the pH range of 3.5 to 4.5 for 60 min.

Key words: *Fusarium subglutinans*, Rennin, Enzyme.

Introduction

In the production of cheese, it is necessary to coagulate the milk in order to separate the casein from the whey. Limited availability of rennin has prompted many investigators in the last decades to search for rennin-like enzyme from microorganisms in general and fungi in particular. Of these fungi, only three strains belonging to *Mucor miehei, M. pusillus* and *Endothia parasitica* are used wide world in production of microbial rennet (Crueger and Crueger 1989; Karlikanova *et al* 1990; Jiao *et al* 1992; Seker *et al* 1998 and Beyenal *et al* 1999).

Other filamentous fungi are recorded as good producers for this enzyme i. e. *Absidia cylindrospora* (Abdel-Fattah *et al* 198 and Ismail *et al* 1987), *Fusarium moniliform* (Kolaczkowska *et al* 1985 and 1988), *Penicillium expansum* (Mabrouk *et al* 1976; Abdel-Fattah and Amr 1987) and *P. oxalicum* (Hashem 1999).

In this work, potentiality of *Fusarium subglutinans* (Wollenweber & Reinking) Nelson *et al* a as good producer for an extracellular rennin-like enzyme has been elucidated. Precipitation of the enzyme from the cell-free dialysate as well as some important properties of the partially purified enzyme were also investigated.

Materials and Methods

Organism. F. subglutinans (Wollenweber & Reinking) Nelson *et al* [det./ 296 RAS] used throughout this work was previously isolated from soil sample collected from Egypt and identified by Centraalbureau Voor Schimmelcutures, Netherlands. The fungus was grown at 30°C on solid Czapek's medium, kept refrigerated and monthly subcultured.

Medium and cultivation. The fermentation medium used at the beginning of the work was that recommended by Wang *et al* (1969) containing 2% (w/v) whole wheat flour in water. Triplicate sets of 250 ml Erlenmeyer flasks were used. Each flask was charged with 50 ml of the fermentation medium. The flasks were sterilized, left to cool, inoculated with equal volumes of spore suspension obtained from 7-day-old cultures and incubated statically at 30°C. At the end of incubation period, each group of flasks were filtered, their contents were mixed and completed to 150 ml with sterilized distilled water.

Assay of milk-clotting activity (MCA). MCA of the enzyme preparation was determined by the method described by Kawai and Mukai (1970) and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1 ml of milk in 40 min at pH 6.0 and 50°C.

*Assay of proteolytic activity (PA).*The PA was assayed by using the procedure of Nomato and Narashi (1959). One protease unit was defined as the amount of enzyme which produce TCA-soluble fragments equivalent to 1µg tyrosine min^{-1} at 40°C.

Enzyme precipitation. Cell-free filtrate (CFF) was dialyzed overnight at 4°C against several changes of 0.1M sodium phosphate buffer (pH, 6.0). Pulverized ammonium sulphate was slowly added to such dialysate to obtain final

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concentration of 0.5-1.0 saturation. Various cooled organic solvents were added separately in another treatment to the dialysate at different ratios. All treatments were left overnight at 4°C and the resulted precipitate were collected and resuspended in phosphate buffer (pH 6.0).

Protein assay. The protein content was measured by the Folin Ciocalteau phenol method suggested by Lowry *et al* (1951).

Results and Discussion

Rennin-like enzyme suitable for cheese making must be characterized with a high MCA and low PA. Importance of this balance (MCA/PA) has been reported by many workers. Effect of culture condition was studied aiming at realizing this hard equation. *F. subglutinans* was found to produce the highest MCE amounting to 648.65 units ml^{-1} at initial pH 4.5 when grown statically (Fig. 1). Ratio of MCA/PA achieved its maximum at the same pH value. The acidic pH value was recorded by other investigators to support the production of rennin-like enzymes from fungal origin (Higashio and Yoshioka 1982; Hegazi 1983; Kolackwska *et al* 1988; Jiao *et al* 1992; Hashem 1999).

Growth at different incubation temperature (Fig. 2) affect the behavior of the fungus under investigation. At 30°C most of the metabolic activities were in a balanced state to push the fungus to produce the highest MCE as well as the optimum MCA/PA. These results are in good agreement with those of Foda (1982); Erdelyi and Kiss (1984) and Jiao *et al* (1992).

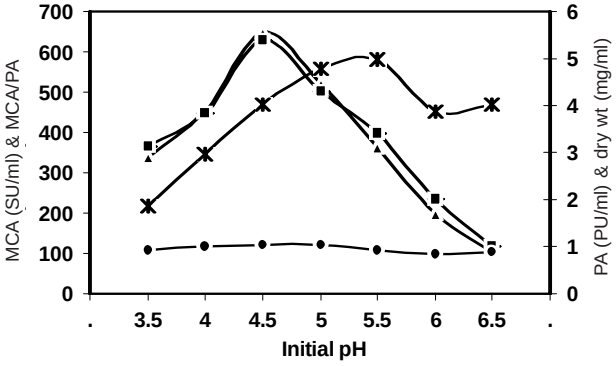

Fig 1. Milk clotting activity, proteolytic activity and dry weight of *F. subglutinans* over pH range 3.5 to 6.0 after 7 days of incubation at 30°C in wheat flour (2%).

—▲—, Milk clotting activity "MCA"; —●—, proteolytic activity "PA"; —■—, MCA/PA; —⁎—, dry wt.,

(Each point represents the mean of 3 measurements; solutions of 0.1 M NaOH and HCl were used for adjustment of initial pH values.)

Production of the extracellular MCE by *F. subglutinans* was followed at different periods of incubation (Fig. 3). The data reveal that the maximum MCE production as well as the MCA/PA were obtained after 6 days of static incubation. At that time, the fungal growth was still active. This indicates that the MCE production by *F. subglutinans* is a growth-associated type. The results further corroborates the previous findings of Iwasaki *et al* (1967); Erdelyi and Kiss (1981); Kolaczkowska *et al* (1988); Jiao *et al* (1992) and Seker *et al* (1998) on extracellular MCE from other fungal sources.

Precipitation of MCE from the culture filtrate of *F. subglutinans* was tested using $(NH_4)_2SO_4$ in addition to some low molecular weight alcohols and acetone in the saturations and ratios illustrated in Table 1. It is clearly evident that iso-propanol (1:1, v/v) precipitate a protein fraction with MCA of 184 SU (yield of 28.37%) at the time that PA showed its least value

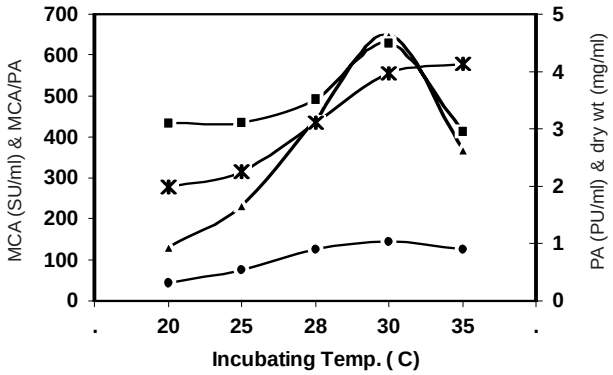

Fig 2. Temperature-dependent variation of milk clotting and proteolytic activities and dry wt of F. subglutinaus.

—▲—, MCA; —●—, PA; —■—, MCA/PA and dry wt; —⁎—, dry wt.; (pH was initially adjusted to 4.5.)

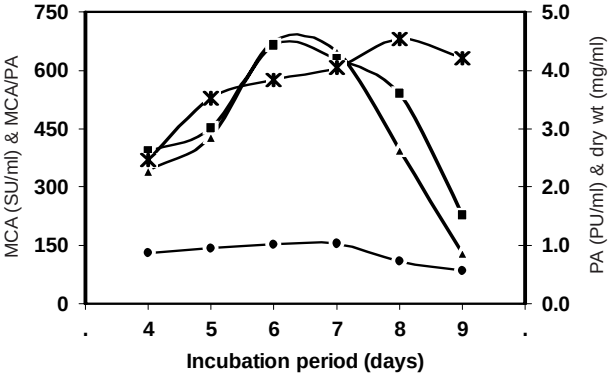

Fig. 3 Time course of milk clotting and proteolytic activities and dry wt of *F. subglutinans*.

—▲—, MCA; —●—, PA, —■—, MCA/PA, in —⁎—, dry wt. (Initial pH 4.5, initial temperature 30°C.)

Table 1
Comparison between different agents used for precipitation of extracellular rennin-like enzyme from *F. subglutinans*

Sample	Ratio (Precipitating agent : CFD) or saturation	MCA (SU)	Yield of MCA (%)	PA (PU)	MCA/PA	% of present (MCA/PA)/ original (MCA/PA)	Total protein (mg/ml)	Specific activity (SU/mg protein)	Purification fold(s)
Cell-free filtrate (CFF)		648.5	100.0	1.00	629	110	100	5.88	1
Cell-free dialysate (CFD)		599.19	100.0	0.92	599	110	95.260	5.43	0.92
Ammoni-um sulfate	0.5	368	56.7	0.480	767	122	22.63	16.26	2.77
	0.6	480	74.0	0.492	976	155	25.17	19.07	3.24
	0.7	560	86.4	0.378	1481	236	30.72	18.23	3.10
	0.8	488	75.3	0.331	1474	234	43.52	11.21	1.91
	0.9	412	63.5	0.288	1431	227	46.70	8.82	1.50
Methanol	1:1	116	17.9	0.102	1137	181	9.73	11.92	2.03
	2:1	124	19.1	0.223	556	88	16.64	7.45	1.27
	3:1	136	21.0	0.395	344	55	27.20	5.00	0.85
	4:1	104	16.0	0.204	510	81	35.16	2.96	0.50
	5:1	96	14.8	0.180	533	85	46.15	2.08	0.35
Ethanol	1:1	96	14.8	0.320	300	48	16.36	5.87	0.99
	2:1	104	16.0	0.297	350	56	19.72	5.27	0.90
	3:1	120	18.5	0.22	545	87	26.10	4.60	0.78
	4:1	100	15.4	0.17	588	94	37.23	2.69	0.46
	5:1	92	14.2	0.12	767	122	45.02	2.04	0.35
Iso-Propanol	1:1	184	28.4	0.10	1840	293	13.71	13.42	2.28
	2:1	252	38.9	0.181	1392	221	16.10	15.65	2.66
	3:1	284	43.8	0.22	1291	205	31.94	8.89	1.51
	4:1	200	30.8	0.31	645	103	37.25	5.37	0.91
	5:1	168	25.9	0.27	622	99	49.00	3.43	0.58
Acetone	1:1	248	38.2	0.386	642	102	20.56	12.06	2.05
	2:1	292	45.0	0.30	973	155	24.13	12.10	2.06
	3:1	360	55.5	0.312	1154	183	36.16	9.96	1.69
	4:1	268	41.3	0.266	1008	160	47.00	5.70	0.97
	5:1	216	33.3	0.215	1005	160	53.59	4.03	0.69

(0.10). This resulted in the highest MCA/PA (1840.0) and the highest % of MCA/PA (292.527) as compared to the original MCA/PA. In this way, the partial purification of this enzyme recorded 2.283 folds. It is worthy of mentioning that ammonium sulfate (0.7 sat) afforded absolutely the highest MCA (560 SU/ml, yield 86%), yet, it allowed so high PA (0.378 PU/ml) resulting in reduction of MCA/PA ratio only to 1481.481. This value represent 80.5% as compared to that obtained with iso-propanol 1:1 (1840.0). It is not in the sake of application to use the fraction obtained from ammonium sulfate. This is totally attributed to the difference in behavior of PA and MCA toward two different precipitating agents.

Influence of both pH and temperature upon the MCA of enzyme preparation was also studied recording 6.0 and 55°C as optima, respectively (Fig. 4). This is in complete agreement with Abdel-Fattah *et al* (1972) who recorded 6.0 as optimum value for activity of MCE from *P. citrinum*. Meanwhile, more acidic range (pH 3.5) was recorded for *Mucor pusillus* by Iwasaki *et al* (1967). Optimum temperature for MCA of enzymes from other microbial sources were found range between 40-45°C for *F. moniliforme* (Kolaczkowska *et al* 1985), 52°C for *A. niger* (Osman *et al* 1969), 60°C for *P. citrinum* (Abdel-Fattah *et al* 1972) and 65°C for *M. pusillus* (Iwasaki *et al* 1967) and *P. oxalicum* (Hashem 2000).

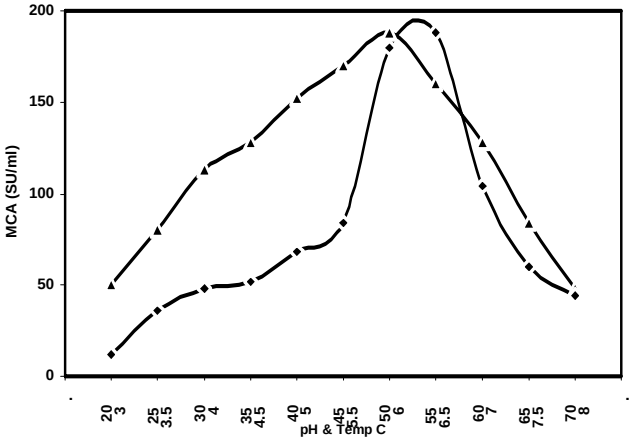

Fig 4. Effect of ptt and temperature upon milk clotting activity "MCA" of partially purified extracellular rennin-like enzyme of *Fusarium subglutinans.*

(—▲—) pH value; (—◆—) temperature.

From the effects of pH value and temperature on stability of the partially purified rennin-like extracellular enzyme from *F. subglutinans*, it can be concluded that generally effect is time dependent and more loss in activity was always recorded with higher time of exposure. The enzyme preparation was held at various pH values (range from 3 to 8) and temperature (range from 15 to 85) for 15, 30, 45 or 60 minutes. Assays was carried out at pH 6.0 and 55°C. It was clear that the effect is time-dependent where more loss in activity was always recorded with higher time of exposure. The enzyme preparation was stable in the pH range of 3.5-4.5 after one hour of exposure, a result not so far from that recorded by Abdel-Fattah *et al* (1972) for stability of enzyme from *P. citrinum* (~ pH 5.0). Moreover, enzyme preparation was completely stable up to 50°C after 1 h of exposure. This means much more stability than that of preparation of *P. oxalicum* where it losses 20% of its activity after 20 min of exposure to 50°C (Hashem 2000). Osman *et al* (1969) found that enzyme preparation of *A. niger* was inactivated at 60°C after 30 min of exposure.

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