Short Communication

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INVESTIGATIONS ON THE ELECTROPHORESIS AND THE ACTIVITY OF THE RENNIN-LIKE ENZYME FROM *ASPERGILLUS NIGER*

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A great deal of work has been carried out on rennin enzymes from animal and plant origins. In recent years, great attention has been directed towards studies on milk-clotting enzymes from microorganisms as rennin substitutes (1–4). In this respect, although some informations about such microbial rennin-like enzymes are now available, yet they are still scanty. In the course of studies on fungal rennin-like enzymes in our laboratory, the production of a milk-clotting enzyme by *Aspergillus niger* No. 58 has been reported, with the effect of some factors on the activity of the crude enzyme preparation (5). In addition, in a further study mainly on the course of proteolysis of the pure enzyme preparation, some properties of the enzyme have been stated (6). The present report concerns with further informations on the electrophoretic separation of the rennin-like enzyme of the afore-mentioned local fungus and the conditions influencing its activity and the milk-clotting process.

The cultivation medium used consisted of corn steep liquor (2%) and lactose (1%). The cultures were made in 250-ml flat-bottomed flasks, each containing 50 ml of sterile medium and the inoculated flasks were incubated at 30° for 3 days. The enzyme powder was obtained by treating the cooled culture filtrate with 2 volumes of ice-cold acetone and the precipitate was dried in a vacuum oven overnight. Paper electrophoresis of the enzyme preparation was performed, with different buffer solutions, using Elphor apparatus and applying a potential of 300 V at room temperature (30°). In each electrophoretic process, the separated bands were cut from the electropherograms and eluted separately with a definite volume of 0.02 M acetate buffer (pH 4.0). The protein content of the enzyme sample was determined by the method of LOWRY *et al.* (7). The milk-clotting activity was determined according to the method of BERRIDGE (8), using a reconstituted skim milk

Table 1. Effect of substrate (milk) concentration on the activity of the rennin-like enzyme of *Aspergillus niger*.

Substrate concn. (g/100 ml)	CaCl ₂ (g/100 ml)	mg Protein/2.5 ml enzyme solution	Time of clotting (min)	Enzyme activity (unit/mg protein)
12	0.11	0.56	17	1.05
14			26	0.68
16			28	0.63
18			30	0.59
20			40	0.44
25			55	0.32
30			55	0.32
12	0.11	0.70	13	1.10
14	0.13		14	1.02
16	0.15		19	0.75
18	0.17		22	0.65
20	0.19		23	0.62
25	0.23		23	0.62
30	0.28		23	0.62

as the substrate. One unit of the enzyme activity was considered to be that which clotted 10 ml of milk in 10 min at 40°.

Electrophoresis of the enzyme sample with 0.2 M phosphate buffer (pH 6.0) did not show any separation. On the other hand, with 0.02 M Veronal buffer (pH 8.0) the enzyme sample shifted towards the anode and separated into 3 components. The band nearer to the origin formed, however, most of the applied material. None of these 3 bands exhibited milk-clotting activity, probably due to the harmful effect brought about by the relatively high pH of the buffer during the electrophoretic process. With acetate buffer the efficiency of separation depended to a great extent on the molarity of the buffer. Thus, 0.2 M acetate buffer (pH 4.0) did not effect any separation of the enzyme sample. On the other hand, with 0.02 M acetate buffer of the same pH, 4 bands separated and shifted towards the cathode. In a previous work (6), the band nearer the origin was proved to be the rennin-like enzyme fraction and thus it was used in the present investigation.

The increase of the substrate concentration in the reaction mixture was associated with a decrease in the milk-clotting activity whether the concentration of calcium chloride was kept constant or proportionally increased with the increase of milk concentration (Table 1). These results reflect a limitation on the enzymic stage and not on the second physicochemical stage of the milk-clotting process. Rise in temperature of the reaction just above 52° considerably reduced the enzyme activity and complete inactivity occurred

Table 2. Effect of some activators and inhibitors on the activity of the rennin-like enzyme of *Aspergillus niger*.

Substance* added	Molarity of substance in enzyme solution	Time of clotting (min)	Enzyme activity (unit/mg protein)	Relative activity %
Control	—	12.12	1.10	100.0
Iodoacetic acid	0.001	12.12	1.10	100.0
	0.010	20.42	0.44	40.0
Maleic acid	0.001	13.48	0.96	87.3
	0.010	19.30	0.68	61.8
Ascorbic acid	0.001	13.00	1.03	93.6
	0.010	17.30	0.76	69.1
Potassium cyanide	0.001	14.48	0.90	81.8
	0.010	16.12	0.82	74.5
Mercuric chloride	0.001	16.12	0.82	74.5
	0.010	16.48	0.79	71.8
Cysteine hydrochloride	0.001	12.42	1.04	95.5
	0.010	24.60	0.55	50.5
Iodine	0.001	9.0	1.48	134.5
Copper sulphate	0.001	9.3	1.40	127.3
	0.010	8.3	1.60	142.6

* The buffered enzyme solution (0.02 M acetate buffer, pH 4.0) was incubated with each substance at 40° for 15 min and then the mixture was added to milk.

when the reaction was conducted at 58°. On the other hand, a slight increase in the pH of the reaction mixture of 0.05 unit than the optimum (5.8) was found to bring about a sharp decrease in enzymic activity. Such effect was, however, considerably influenced by the temperature of the reaction. In this respect, the enhancement effect of temperature ceased whenever pH of the reaction mixture reached 6.1.

Concerning the effect of treating the enzyme solution with some agents on the clotting of milk (Table 2), it was found that iodoacetic acid, maleic acid, mercuric chloride, potassium cyanide, cysteine hydrochloride, and ascorbic acid caused retardation in the time of clotting. On the other hand, treatment of the enzyme with iodine or copper sulphate before its addition to milk enhanced clotting. The inhibition of rennin action by mercuric chloride was also reported by REGE (9) and BERRIDGE (10). On the other hand, activation of the enzyme brought about by treatment with iodine may be the reason for its use as a sterilising agent for rennin (11). It is worthy to mention that,

although the comparatively low activation or inhibition by the various agents used does not justify elaborate indulgence in the explanation of their stimulatory or inhibitory effect, the results obtained collectively refer to the absence of sulfhydryl groups from the active site of the rennin-like enzyme in question. However, it should be pointed out that, during the renneting process, changes in the casein complex are brought about (9) which may interfere with such agents.

REFERENCES

- 1) T. TSUGO, U. YOSHINO, K. TANIGUCHI, A. OZAWA, Y. MIKI, S. IWASAKI and K. ARIMA, *Japan J. Zotech. Sci.*, **35**, 221 (1964).
- 2) I. YA. VESELOV, D. YA. TIPOGRAPH and T.A. PETINA, *Prikl. Biokhim. Microbiol.*, **1**, 52 (1965).
- 3) S.G. KNIGHT, *Can. J. Microbiol.*, **12**, 420 (1966).
- 4) S. IWASAKI, G. TAMURA and K. ARIMA, *Agric. Biol. Chem. (Tokyo)*, **31**, 546 (1967).
- 5) H.G. OSMAN, A.F. ABDEL-FATTAH, M. ABDEL-SAMIE and S.S. MABROUK, *J. Gen. Microbiol.*, **59**, 125 (1969).
- 6) H.G. OSMAN, A.F. ABDEL-FATTAH and S.S. MABROUK, *J. Gen. Microbiol.*, **59**, 131 (1969).
- 7) O.H. LOWRY, N.J. ROSEBROUGH, A.L. FARR and R.J. RANDALL, *J. Biol. Chem.*, **193**, 265 (1951).
- 8) N.J. BERRIDGE, *Analyst*, **77**, 57 (1952).
- 9) U.Y. REGE, *Dissertation Abstr.*, **24**, 1825 (1963); Cited from *Dairy Sci. Abstr.*, **26**, No. 4 (1964).
- 10) N.J. BERRIDGE, *The Enzymes*, Vol. 1, Academic Press, Inc., New York (1951), p. 1096.
- 11) J.G. DAVIS, *Cheese*, Vol. 1, J. & A. Churchill Ltd., London (1965), p. 250.