USE OF PROTEASES OF MICROBIAL ORIGIN IN THE DAIRY INDUSTRY*

II. EXPERIMENTS TO PRODUCE A MILK-COAGULATING ENZYME PREPARATION FROM CULTURES OF ENDOTHIA PARASITICA AND OF MUCOR PUSILLUS[1]

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Introduction

The increased use of milk-coagulating enzyme preparations of microbial origin necessitates studies to find methods for the precipitation of the enzyme from the fermentation liquor at the minimum loss of enzyme possible. In general, the enzyme used to be extracted from the solid bran cultures with water or with solutions of sodium chloride[1], and the obtained extract is treated with methanol, ethanol [2] or ammonium sulphate to precipitate the enzyme which is dried in vacuum or freeze-dried. Since data of literature available to us were very scarce, it was our aim to establish the optimum conditions of precipitation by experiments.

Experimental material and methods

On completing the fermentation process, the methods generally used in literature were tested first. The precipitate obtained in this way was centrifuged, resolved with distilled water to its initial volume, its milk-coagulating capacity determined [6] and expressed as percentage of the activity of the original fermentation liquor.

Precipitation experiments were performed with the precipitating agents found to be the most suitable to establish the dependence of precipitation on the concentration, on pH values and on temperature. Since our preliminary tests proved that the fermentation liquors of both Endothia parasitica and Mucor pusillus very quickly lose their activity at alkaline pH values, our studies have been limited to the acidic pH domains.

The homogeneity of the enzymes precipitated with ethanol and ammonium sulphate was checked by paper electrophoresis [4]. A 10% solution of the enzymes in an appropriate buffer was dialyzed against the applied buffer for

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two days. Runnings were carried out for 7 to 9 hours at a voltage of 180 V. For the investigations the following buffer solutions were used:

	pH
1. 0.1 *M* phosphate	1.95
2. 0.1 *M* phosphate	3.50
3. 0.1 *M* phosphate	4.78
4. 0.1 *M* phosphate	6.52
5. 0.1 *M* phosphate	7.00
6. 0.1 *M* phosphate	8.57
7. 0.1 *M* veronal-sodium acetate	8.60
8. 0.1 *M* veronal in 40% urea	8.60
9. 0.1 *M* veronal in 40% urea	9.50
10. 0.1 *M* phosphate in 40% urea	8.60
11. 0.1 *M* phosphate in 40% urea	7.50
12. 0.1 *M* phosphate in 40% urea	7.00
13. 0.1 *M* phosphate in 40% urea	5.44

The obtained electropherograms were evaluated by means of the automatic evaluator ERI [4].

Experimental results and their evaluation

1. *Investigation of the conditions of precipitation*

1.1. The data of our research into the conditions of precipitation of the milk-coagulating enzyme produced by *Endothia parasitica* are presented in Table 1.

Table 1

Recovery of milk-coagulating activity from the fermentation liquor of Endothia parasitica

Precipitating agent	Terminal concentration of agent	Temperature of precipitation, °C	Yield, %, at pH 2	pH 4	pH 6	pH 7
Ammonium sulphate	40	20	69	20	6	15
	50		78	44	37	34
	60		82	71	48	51
	70		67	75	52	48
Ethanol	60	10	0	5	40	18
	70		0	73	70	51
	80		0	71.5	73	55
	85		0	74	72	55
	70	20	0	72	70	45
		30	0	27	64	14
		40	0	5	35	0

It is seen from the data in Table 1 that on using ammonium sulphate as precipitating agent, the best results were obtained in general at a terminal concentration of 60%, and the yield was not unequivocally improved by the further increase of the concentration of precipitant. In the acidic domain (pH 2—4) the precipitate showed always higher activities than at pH 6—7 approaching neutrality. On using ethanol, the increase of the terminal concentration to over 70% did not rise the activity any further. On precipitation with ethanol, the domain of pH 4—6 proved to be the most favourable since in a more acidic medium (pH 2) the enzyme could not be precipitated at all. No differences in precipitation results at +10 °C and +20 °C were observed. In general, a marked decrease of activity was experienced at higher temperatures.

1.2. The bran culture of *Mucor pusillus* was rubbed with a fivefold volume of distilled water. For our investigation the mixture was filtered or centrifuged. It must be noted that only about 50% of the activity can be recovered by one single aqueous extraction.

The experimental data concerning the conditions of precipitation of the milk-coagulating enzyme produced by *Mucor pusillus* are given in Table 2.

Table 2

Recovery of milk-coagulating activity from the fermentation liquor of Mucor Pusillus

Precipitating agent	Terminal concentration of agent	Temperature of precipitation, °C	Yield, %, at			
			pH 2	pH 4	pH 6	pH 7
Ammonium sulphate	40	20	36	35	10	12
	50		31	60	58	40
	60		85	82	68	46
	70		51	33	60	50
Ethanol	60	10	0	40	11	7
	70		0	73	65	51
	80		0	73	65	50
	85		10	74	65	51
	70	20	0	74	63	49
		30	0	73	65	47
		40	0	74	67	24
Acetone	50	10	0	0	0	0
	60		0	69	45	53
	70		0	71	66	51
	60	20	0	67	65	49
		30	0	62	64	25
		40	0	60	65	0

From the data presented in Table 2 it appears that, similarly to the enzyme of *Endothia parasitica*, the best result on using ammonium sulphate was obtained in the domain pH 2—4 at a terminal concentration of 60%. The unfavourable effects of applying concentrations of ammonium sulphate higher than 60% are even more pregnant in the case of the enzyme produced by *Mucor pusillus*. On using ethanol, yields could not be raised by increasing the ethanol concentration over a terminal concentration of 70%. Also in that case the pH domain 4 to 6 proved to be the most suitable while at pH 2 the enzyme could be hardly precipitated if at all. On investigating the dependence of precipitation on temperature, it can be stated that in general the activity does not decrease up to 30 °C. The highest sensitivity of the enzyme to heat was observed at pH 7. The scattering of the precipitation reactions was found not to be significantly higher than that of the determination of coagulating activity used as fundamental method [8, 9].

2. *Electrophoretic investigations*

2.1. Data of the electrophoretic investigation of the coagulating enzymes of *Endothia parasitica* are presented in Table 3.

Table 3

Data of the electrophoretic investigation of the coagulating enzymes of Endothia parasitica

Number of buffer	Number of precipitated protein fractions	Precipitant applied	Percentages of fractions from start point	
			nearer	farther
4	1	ethanol		
4	2	ammonium sulphate	73.4	26.6
5	1	ethanol		
5	2	ammonium sulphate	78.1	21.9
7	1	ammonium sulphate		
8	1	ammonium sulphate		
9	1	ammonium sulphate		
10	1	ammonium sulphate		
11	2	ammonium sulphate	58.9	41.1
12	2	ammonium sulphate	53.7	46.3
13	1	ammonium sulphate		

In buffers 3 and 6 the enzymes could not be got running.

In buffers 8, 9, 10, 11, 12 and 13, the enzyme precipitated with ethanol could not be get running.

In the coagulating enzyme preparation precipitated with ethanol only one fraction was obtained with every running agent tested. On applying am-

monium sulphate as precipitant, in some cases two fractions were observed the amount of which in urea as medium essentially differed from the area ratios obtained in pure phosphate buffer. On taking these observations into account we considered the precipitation by ethanol to be more appropriate for the production of a homogeneous enzyme preparation.

2.2. Data of the electrophoretic investigation of the coagulating enzymes of *Mucor pusillus* are given in Table 4.

Table 4

Data of the electrophoretic investigation of the coagulating enzymes of Mucor pusillus

Number of buffer	Number of precipitated protein fractions	Precipitant applied	Percentage of fractions		
			near to start point	in the middle	farther from start point
1	2	ethanol	58.7	—	41.3
1	2	ammonium sulphate	56.0	—	44.0
2	2	ethanol	54.2	—	45.8
2	2	ammonium sulphate	52.2	—	47.8
3	2	ethanol	54.8	—	45.2
3	2	ammonium sulphate	61.5	—	38.5
4	3	ethanol	44.1	30.9	25.0
4	3	ammonium sulphate	36.8	31.7	31.5
5	2	ethanol	61.7	—	38.3
5	2	ammonium sulphate	56.7	—	43.3
6	2	ethanol	53.5	—	46.5
6	2	ammonium sulphate	52.7	—	47.3

It is seen from the results of investigations carried out at different pH values that on using ethanol and ammonium sulphate, respectively, as precipitants, the same fractions are obtained. It is of interest to note that at the pH value 6.52, fraction ratios and distributions quite deviating from all others have been obtained. As it was expected on the basis of the data of investigation by precipitation and by electrophoresis, the activity of the dry enzyme preparations varied from 20 000 to 30 000, independently of the mode of precipitation and of the cultured strain. This means an enrichment by about 200, referred to the enzyme amount present in the fermentation liquor.

Summary

The optimum conditions of precipitation of the milk-coagulating enzyme from the fermentation liquors of *Endothia parasitica* and *Mucor pusillus* strains were established, and the purity of the enzymes recovered in various ways was investigated by paper electrophoresis.

It was found that in the case of *Endothia parasitica* the highest yields were obtained on applying a terminal concentration of 60% of ammonium sulphate (at pH 2 to 4) or of 70% of

ethanol (at pH 4 to 6). The loss of activity was nearly the same in both cases up to a temperature of +20 °C. In the case of *Mucor pusillus*, also a terminal concentration of 60% of ammonium sulphate (at pH 2 to 4) or of 70% of ethanol (at pH 4 to 6) was found to be the most appropriate. In the acidic domain, no significant decrease of activity was observed up to 30 °C.

According to the electrophoretic investigations, the enzyme of *Endothia parasitica* precipitated with ethanol is purer than that precipitated with ammonium sulphate. In the case of the enzyme of *Mucor pusillus*, the enzyme preparations precipitated with the tested two precipitants showed an electrophoretically almost identical composition.

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