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STUDIES ON ACYLASE

II. PURIFICATION OF ACYLASE

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Many investigations (1–5) have been made on the specificity, distribution, purification and so on of acylase. A partial purification of acylase, especially the differentiation from dipeptidase was reported previously (6). In the present work an effort of further purification of acylase was made and the purification of about 50-fold was attained.

EXPERIMENTAL

Material: Pig kidney which was used as the source of enzymes, was treated in the same way as described in the previous report. It was first washed with water, removed of fat and connective tissue and homogenized with 2 to 3 times its volume of water. The homogenate was centrifuged at about 3,000 r. p. m. for 20 to 30 minutes. The obtained turbid supernatant was referred to as the "original extract".

Experimental methods were the same as described previously. Digestion mixture consisted of 2 ml. of enzyme solution, 5 ml. of 0.066 M phosphate buffer of pH 7.0 and 8.0 for acylase and dipeptidase respectively, 10ml. of 0.1 M neutralized substrate solution and 8 ml. of water. As the control served the mixture wich was added with 10 ml. of water instead of substrate solution. The enzymatic hydrolysis of the substrate was followed by the formol titration, after the incubation of definite time at 37°C. Acidity increase of 5 ml. mixture was determined with 0.1N NaOH.

In the course of the purification of acylase, specific activity was measured as follows: To the combined solution of 1.0 ml. of 0.2M neutralized substrate solution, 0.5 ml. of 0.066 M phosphate buffer of pH 7.0 for acylase and 8.0 for dipeptidase and 0.5 ml. of water, was added 0.5 ml. of enzyme solution or distilled water as control. After incubation at 37°, the digstion mixture was formol-titrated with 0.1 N NaOH. 2ml. of acidity increase corresponded to 100 per cent hydrolysis of the substrate. Titration values under 1.00 ml. (50 per cent hydrolysis) were used for the calculation of the specific activity, assuming that the hydrolysis were liner versus time up to 50 per cent. The unit of specific activity was chosen arbitrarily and expressed with the value of acidity increase per hour per mg. nitrogen in the enzyme solution used.

The nitrogen was measured with the usual micro-Kjeldahl method.

The calcium phosphate gel for the adsorption was prepared according to *Kunitz*(8).

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The electrophoresis-convection was carried out according to *Cann* et al (9) with an apparatus made by *Nakamura*. The volumes of the top and the bottom reservoir were 25 and 50 ml., respectively.

Electrophoresis was carried out according to the usual method of Tiselius with a halfmicro cell of 1.5ml. capacity.

A. PRELIMINARY EXPERIMENTS

In order to work out the procedure of purification, various preliminary experiments were at first performed.

(1) Acid fractionation.

Four portions of the original extract were adjusted to pH 4.0, 4.5, and 4.7, respectively, by the addition of 1 N HCl in the cold and centriufged. The clear redish yellow supernatants were neutralized and their enzymatic activities were measured.

Table I. Activities of Acid Fractions from the Original Extract of Pig Kidney.

pH of Acid fraction	Incubation time in hours	Acidity increase in 5 ml. digestion mixture (ml. 0.1 N NaOH).	
		Acetylglycine	Diglycine
Original Extract	1.5	0.93	1.73
	3	1.70	1.87
	6	1.91	1.93
4.0	1.5	0.18	0.90
	3	0.43	1.52
	6	1.07	1.86
4.5	1.5	0.47	1.12
	3	1.20	1.76
	6	1.82	1.90
4.7	1.5	0.80	0.62
	3	1.60	1.21
	6	1.94	1.80

As to the experimental conditions, cf. text.

As can be seen from the results shown in Table I, in the supernatant of pH 4.7 remained almost all the acylase activity presented in the original extract, whereas in that of pH 4.0 considerable inactivation occurred.

The particulates contained in the original extract could only be separated by the prolonged high speed centrifugation, as previously reported (6). The turbidity, however, could be very easily eliminated by the acidification, together with the precipitate formed thereby. But if the pH attained was higher than about 5, the elimination of turbidity was not sufficient. Therefore the very favourable circumstance can be utilized for the purification of acylase, that by the acidification to pH 4.7 the clear supernatant could be obtained without the loss of activity.

(2) Acetone fractionation.

In order to test the effectiveness of acetone fractionation (7), following experiments were carried out: Ice cold acetone was added under vigorous stirring to five portions of original extract to make the concentration in acetone, 30, 40, 45, 50, and 55 volume per cent, respectively. Each solution was centrifuged and the turbid supernatant was filtered in a refrigerator. The filtrate obtained was diluted 10 times with water and submitted to the measurement of enzyme activity. From the results given in Table II, it will be seen that the acylase remained chiefly in the supernatant, when the concentration of acetone was smaller

Table II Activities of Supernatant of Acetone Precipitation.

Percetgae of acetone in the supernatant	Incubation time in hours	Acidity increase in 5 ml. digestion mixture (ml. 0.1 N NaOH)	
		acetylglycine	Diglycine
30	6	1.28	0.39
	9	1.69	0.57
	24	1.78	1.04
40	4	0.71	0.24
	19	1.85	1.14
	28	1.86	1.29
45	5	0.15	0.00
	20	1.52	0.34
	40	1.87	0.62
50	20	0.80	0.20
	44	1.51	0.36
55	20	0	0
	44	0	0
	92	0	0

than 40 volume per cent. On the other hand it was precipitated totally, if the concentration in acetone reached 55 per cent.

Acetone fractionation is favourable in that the precipitate can be readily freed from the solvent used and extracted with small quantity of suitable solvent, making possible to concentrate the enzyme. This procedure, however, is unfavourable to be utilized in the first step of purification, since a clear supernatant can not be obtained without sacrificing the enzyme activity.

(3) Fractionation with ammonium sulfate.

As already mentioned above, the acid extract, namely the acid fraction obtained by acidifying the original extract, was very favourable as the starting enzyme solution for the further purification. Thus it was utilized in the experiments of fractionation with ammonium sulfate.

The neutralized acid extract was added with ammonium sulfate at first to 0.35 saturation, then half and total saturation. The precipitates obtained were suspended in a small amount of water and dialyzed against distilled water for 48 hours. After centrifugation the enzymatic activities of the solution was

measured.

The results presented in Table III show that almost all the enzyme activity was retained in the precipitate obtained by 0.35 saturation of ammonium sulfate.

Table III Fractionation of Pig Kidney Extract with Ammonium Sulfate.

Degree of saturation of ammonium sulfate,	Incubation time in hours.	Acidity increase in 5 ml. digestion mixture (ml. 0.1 N NaOH)	
		Acetylglycine	Diglycine
Acid extract	1.5	0.45	0.28
	3	0.64	0.42
	6	1.13	0.81
Precipitate from 0.5–1.0 sat.	24	0	0.31
	72	0	0.59
Precipitate from 0–0.35 sat.	1.5	0.48	0.25
	3	1.12	0.53
	6	1.81	1.04

As to the experimental conditions, cf. text.

It is to be noted here that the redish pigment of the original acid extract was removed by this procedure and the total volume of enzyme solution could be reduced markedly as in the case of acetone fractionation.

(4) Adsorption with calcium phosphate gel.

The enzyme solution used for the experiments of adsorption was the acid extract as in the case of ammonium sulfate fractionation. It was divided into 5 portions and 4 of them were added with the calcium phosphate gel of 0.1, 0.2, 0.5 parts of their volume respectively, as shown in Table IV.

Table IV Effect of Adsorption with Calcium Phosphate Gel.

pH of the adsorbed enzyme solution	Volume of gel added, in	Incubation time, in hours.	Acidity increase in 5 ml. digestion mixture (ml. 0.1 N NaOH)	
			Acetylglycine	Diglycine
4.5	0.1	1.5	0.64	0.42
		3	1.50	1.09
		6	1.97	1.59
4.7	0.2	1.5	0.82	0.37
		3	1.59	0.91
		6	1.96	1.57
4.7	0.5	1.5	0.81	0.25
		3	1.57	0.63
		6	1.93	1.21
7.0	0.5	1.5	0.90	0.55
		3	1.68	1.18
		6	1.94	1.69
Acid Extract		1.5	0.80	0.62
		3	1.62	1.21
		6	1.94	1.80

As to the experimental conditions, cf. text.

The remaining one portion served as control after being neutralized. After 15 minutes of stirring with the gel, they were centrifuged. The supernatants obtained were adjusted to pH 7.0 and if turbid, centrifuged again.

From the results obtained it can be seen that the acylase remained in the rest solution, not being adsorbed by the gel. On the other hand, dipeptidase was to some extent adsorbed at pH 4.7. (Tables IV)

(5) Fractionaton by electrophoresis-convection.

As the capacity of the electrophoresis-convection apparatus was limited, the procedure was intended to utilize in the later step of purification. Thus basing on the experiments described above, the starting material was partially purified as follows. The original acid extract was precipitated with ammonium sulfate at 0.3 saturation and the precipitate obtained was fractionated further with acetone. The fraction obtained at acetone concentration of 30 to 60 per cent was dissolved in water and dialyzed against phosphate buffer of pH 6.0 and ionic strength 0.1 for 24 hours. It was run in the apparatus for 24 hours using the same buffer. At the end of run considerable precipitate appeared.

From the results shown in Table V, the reduction of the enzyme activitites in the

Table V Activities of the Fractions obtained by Electrophoresis-Convection from the Purified Acylase.

Fraction	Incubation time, in min.	Acidity increase in 5 ml. digestion mixture (ml. 0.1 N NaOH)	
		Acetylglycine	Diglycine
Starting Material	60	0.43	0.16
	120	0.88	0.35
	180	1.19	0.49
Top-cut	30	0.09	0.04
	60	0.17	0.08
	120	0.34	0.15
Bottom-cut	30	0.32	0.08
	60	0.67	0.20
	120	1.23	0.41

As to the experimental conditions, cf. text.

top cut can be seen. Even in the bottom reservoir both acylase and dipeptidase were not concentrated. The procedure was reported usually to be very mild in its mode of action. Unfortunately a considerable precipitation of proteins occured during the procedure, indicating the denaturation of the enzymes together with the innert proteins. Thus it was not adopted to the final procedure of purification of acylase, against the initial intention.

B. Purification of Kidney Acylase

As to the procedures adopted, some preliminary remarks may be made: As discussed previously, by the high speed centrifugation of the original extract a fractionation of the acylase and the dipeptidase was possible. But the procedure

was not favourable for the purification of the acylase, since it could not be separated from the dipeptidase notably. Considering also the disadvantage of the limitation in capacity it was not adopted in the procedure of purification.

The precipitation with the acetone fractionation from 30 to 60 per cent of acetone concentration, was chosen to prevent the loss of the enzyme as possible, instead of 50 per cent.

Many other procedures examined preliminarily were not described here, since they were not adopted to the final procedure.

From the preliminary experiments performed, the following procedure of pufication of acylase was established.

About 1 kg. of fresh pig kidneys were freed from fat and connective tissue and homogenized in 150 g. batches in a cooled mixer with 2 times its volume of ice water for 2 to 3 minutes. The homogenate was centrifuged at 3,000 r.p.m. for 20 minutes in a cooled room at about 0°. The supernatant was cooled to 0° and brought to pH 4.7 by the careful addition of 2N HCl under vigorous stirring. The resulting suspension was centrifuged at 4,000 r.p.m. for 20 minutes. The precipitite was discarded and the clear redish supernant was adjusted to pH 7.0 with 2 N NaOH.

It was then precipitated by the addition of solid ammonium sulfate to 0.35 saturation. The pH of the solution changed whereupon to about 6.0. The suspension was allowed to settle in a refrigerator overnight, then the redish supernatant was siphoned off prior to centrifugation. The precipitate obtained by centrifugation weighed about 60 g. and was suspended in 60 ml. of ice water and dialyzed against running tap water until completely free from ammonium sulfate.

The precipitate which appeared during the dialysis was centrifuged off and the clear dark brown supernatant was added with 0.2 parts of its volume of calcium phosphate gel. The suspension was adjusted to pH 5.0 with a dilute acetic acid and stirred for 15 minutes.

The clear supernatant obtained by centrifugation at 3,000 r.p.m. for 10 minutes was precipitated once more with ammonium sulfate and adsorbed by calcium phosphate gel in the same way as before. About 40 ml. of clear brown solution were obtained.

To the solution, acetone was slowly added under 0°C to make 30 per cent by volume and centrifuged. Then the supernatant obtained was cooled to −8° and added carefully with acetone to 60 per cent of the original volume of the solution. After centrifugation, the precipitate obtained was dissolved in 2 ml. of 0.1 M phosphate buffer of pH 7.0 and centrifuged again, if neccessay. About 2.7 ml. of clear yellow solution were obtained.

The procedures of the purification was schematically summarized in Fig. 1.

Fig. 1 Procedure of the Purification of Acylase.

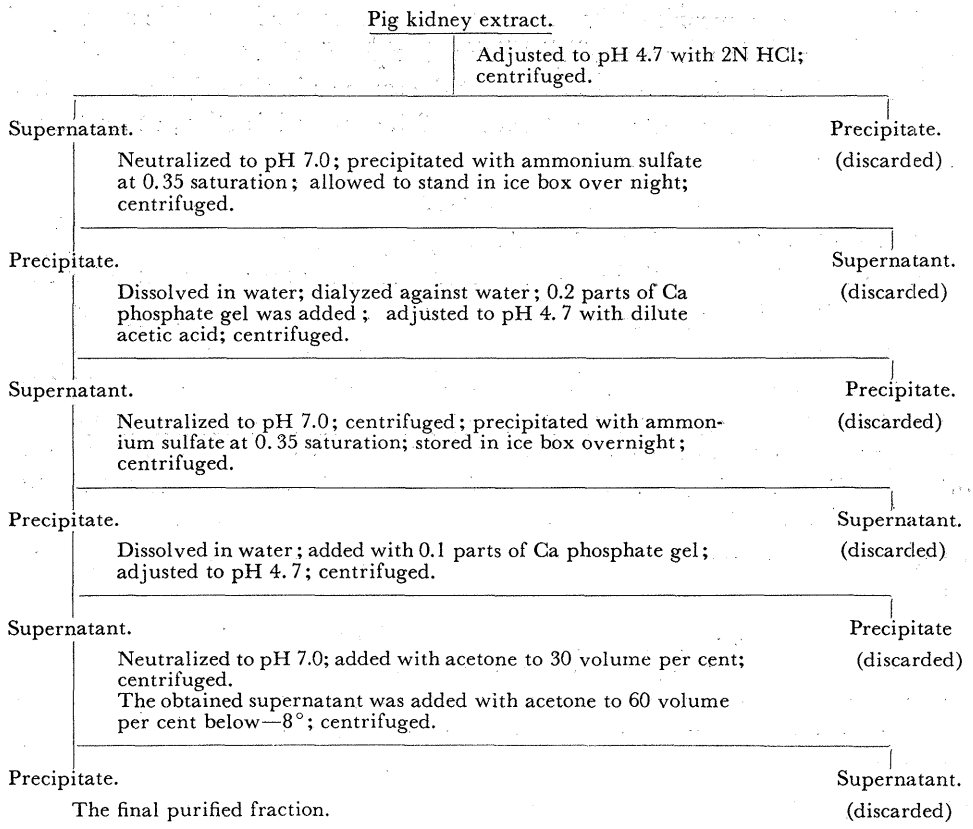

Table VI Specific Activites of the Fractions produced in the Course of Purification.

Fraction in the course of,	Total volume obtained, in ml.	Specific activity; Acidity increase per hour per nitrogen.	
		Acylase	Dipeptidase
Original extract	2080	0.6	4.3
Acid extract	1200	1.4	4.0
Precipitate with ammonium sulfate	160	3.4	5.4
Rest soluion adsorption with Ca phosphate	148	4.5	3.3
Precipitate with ammonium sulfate	32	9.8	7.4
Rest solution, adsorption with Ca phosphate	32	10.3	7.4
Precipitate with acetone	2.7	31.2	1.5

As to the experimental conditions, cf. text.

As shown in Table VI, the over-all recovery of the activity in the final, purified fraction was approximately 10 per cent. The increase in specific activity of acylase per nitrogen was roughly 50-fold, compared with the original extract. The dipeptidase activity remained almost the same as in the original extract.

By the preliminary electrophoretic study of the purified fraction, at least three components were recognized.

SUMMARY.

A procedure for the purification of kidney acylase was presented. It consisted in the repeated fractionation with ammonium sulfate and adsorption of innert proteins with calcium phosphate gel of the acid extract followed by thefractionation with acetone.

By the procedure proposed, about 50-fold purification of the kidney acylase was attained and the over-all recovery of the enzyme was about 10 per cent.

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