Bull. Yamayuchi Med. School, Vol. 5, No.1, 1957

STUDIES ON ACYLASE

I. DIFFERENTIATION OF ACYLASE FROM OTHER PEPTIDASES

Tsutomu HOSODA

Institue of Medical Chemisty, Yamaguchi Medical School, Ube

(Received Jannuary 30, 1957)

For the purification of acylase and peptidases, adsorpion procedures were applied by many investigators (1–8). Starting from both the homogenate and the acetone poweder of pig kidney, they attempted to purify the enzymes, using kieselguhr, $Al(OH)_3$ gel and $Fe(OH)_3$ gel as adsorbent. But a definite separation of kidney acylase and dipeptidase was not attained. In the pig kidney, there can be recognized the enzymatic activities against acetylglycine, benzoylglycine, diglycine, acetyldiglycine and benzoyldiglycine. Considering the similarity of the chemical structure of these substances, there rises the question whether they are hydrolyzed by the same enzyme or not. If one of them could be enriched or reduced by any method independently from other activities, it becomes clear that they rest on different enzymes.

The present work was attempted to differentiate these enzymatic activies as the preliminary experiments of the purification of acylase.

EXPERIMENTAL

Material : As the source of enzyme solution, the pig kidney was used. It was first washed with water, removed of fat and connective tissue, cut into pieces with a knife and then homogenized with 2 to 3 times its volume of cooled distilled water in a mixer. The homogenate was centrifuged at about 3,000 r. p. m. for 20 to 30 minutes. The obtained turbid supernatant was used as the starting material and referred to in the following as the "original extract".

Methods : Each digestion mixture contained 2ml. of enzyme solution, 5 ml. of 0.066M phosphate buffer, 10ml of either 0.1 M neutralized substrate solution (acetylglycine, diglycine etc.) or water as control and 8 ml.of water, making the total volume into 25 ml. The pH of the buffer was 7. 0 for the measurement of acylase, 8. 0 for that of dipeptidase and 7.5 for carboxypeptidase. When the inhibitory effect of a substance was to be tested, 5 ml. of 0.05 M solution of the substance to be tested and 3 ml. of water were added instead of 8 ml. of water.

At a definite time after incubation at 37°C, the activity of 5 ml. of digestion mixture was formoltitrated with 0.1 N-NaOH. The values corrected for controls

細　田　　勉

are shown as the acidity increase in each table. The total hydrolysis of the substrate corresponded to 2 ml. increase of titration values, since the final concentration of the substrate was 0.04 M. In some tables the activity value was also given in percentage of the hydrolysis of the substrates.

Nitrogen was estimated with the usual semimicro Kjeldahl method.

RESULTS AND DISCUSSION

(1) Optimal pH of enzymatic activities of pig kidney extract toward diglycine, acetylglycine and benzoylglycine.

The enzyme solution used for diglycine and acetylglycine was the original extract diluted 30 times with water and that for benzoylglycine was diluted 3 times.

The buffer used were 0.066 M phosphate for pH below 8.0 and 0.1 M borate for pH above 8.3. The results obtained were shown in Table I.

As can be seen from the results, the hydrolysis of both acetylglycine and benzoylglycine by kidney extract took place at pH 7.0 optimally, only the former was hydrolyzed more readily than the latter. Under the same conditions the hydrolysis of diglycine was optimal at pH 8.0 and was most rapid of the three substances tested.

TABLE I

Optimal pH toward Diglycine, Acetylglycine and Benzoylglycine.

Substrate	Digestion time, in hours.	Acidity increase in 5ml. digestion mixture, expressed in ml of 0.1 N NaOH.								
		PH								
		6.0	6.5	7.0	7.5	8.0	8.3	8.5	9.0	9.5
Diglycine	2	—	—	0.28	0.36	0.67	0.76	0.67	0.61	0.11
	5	—	—	0.68	0.89	1.61	1.43	1.39	1.11	0.53
	20	—	—	1.75	2.09	2.18	2.07	2.06	1.56	1.00
Acetylglycine	2	0.20	0.24	0.26	0.16	0.12	—	0.09	0.08	—
	5	0.68	0.86	0.90	0.80	0.63	—	0.46	0.36	—
	20	2.00	2.00	2.00	2.00	2.00	—	1.40	0.97	—
Benzoylglycine	20	0.66	0.76	0.90	0.70	0.70	—	—	0.37	—
	28	0.94	1.09	1.22	1.07	0.95	—	—	0.51	—
	44	1.46	1.68	1.81	1.59	1.52	—	—	0.63	—

As to the experimental conditions, cf. text.

The original extract used as the enzyme solution was diluted with water 30 times for diglycine and acetylglycine, while 3 times for benzoylglycine.

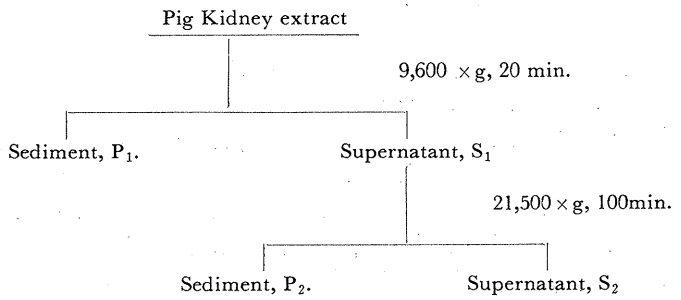
Now that the optimal pH's were established, they were utilized for the following experiments.

(2) Fractionation by high speed centrifugation.

The differentiation of various peptidases in the kidney extract can be effected by many ways. Perhaps the most simple one would be the fractionation by centrifugation, only if it were possible.

The kidney extract was prepared with 5 times its volume of water. Then it was

Fig. 1. Fractionation by High Speed Centrifugation.



centrifuged further for 20 minutes at 9,600 × g at 0°C. Redish brown, turbid supernatant, S_1, and sediment, P_1, were obtained. The former was again centrifuged at 21,500 × g for 100 minutes at 0° and clear redish supernatant, S_2, and

Table II

Fractionation of the Pig Kidney Extract by High Speed Centrifugation.

Fraction	Digestion time in hours.	Acidity increase in 5ml. digestion mixture (ml. 01 N NaOH)	
		Acetylglycine	Diglycine
Original extract	1	0.28	0.71
	2	0.52	1.25
	4	1.00	1.71
S_1	1	0.31	0.48
	2	0.62	0.88
	4	1.04	1.36
P_1	1	0.05	0.45
	2	0.10	0.87
	4	0.22	1.41
S_2	1	0.29	0.18
	2	0.57	0.35
	4	1.04	0.65
P_2	1	0.00	0.31
	2	0.03	0.61
	4	0.05	1.04

sediment, P_2, were obtained. The scheme of preparation is given in Fig. 1. The activities of the fractions obtained, S_1, P_1, S_2, and P_2, together with the

original extract were tested against diglycine and acetylglycine.

The results obtained are shown in Table II. It can be clearly seen from them, that the ratio of activity toward acetylglycine and that toward diglycine is different in each fraction. The ratio is larger in the supernatants and smaller in the sediments. Thus it can be inferred that the acylase activity is located in the water soluble component and the dipeptidase activity on the water insoluble particles of the cell. Hence the two activities can be ascribed to the two different enzymes: acylase and dipeptidase.

(3) Differentiation by acid inactivation.

The original extract was divided into four portions and adjusted under cooling to pH 3.5, 4.0, 4.2 and 4.5 with 1 N HCl respectively, and stored in an ice box for 20 hours. Then they were neutralized to pH 7.0 with 1 N NaOH and centrifuged. The supernatants obtained were submitted to the measurements of their enzymatic activities.

As can be seen from the results shown in Table III, considerable inactivation

Table III

Acid Inactivation of the Pig Kidney Extract.

PH of storage	Digestion time, in hours.	Acidity increase in 5 ml. digestion mixture (ml. 0.1N NaOH)		
		Diglycine	Acetylglycine	Benzoylglycine
Original extract	5	1.61	0.90	—
	20	2.18	2.00	0.77
3.5	20	0.00	0.00	0.00
	68	0.27	0.14	0.00
	116	0.30	0.27	0.00
4.0	20	1.31	0.08	0.00
	44	1.84	0.21	0.00
	92	2.06	0.42	0.06
4.2	20	1.59	0.47	0.05
	44	1.91	0.85	0.14
	68	2.09	1.33	0.32
4.5	20	1.81	1.80	0.35
	44	1.97	1.89	0.92
	68	2.11	1.91	1.44

As to the experimental conditions cf. text.

under pH 4.2 was recognized. The dipeptidase activity, however, was most resistant to the acid inactivation. The acylase activity could not be wholly inactivated, without sacrificing the dipeptidase activity at the same time. So that the two activities can not be fractionated by this way. But it is sufficient to distinguish

them from each other.

The hippuricase activity was almost indetectable at pH 4.0. But as it was essentially weaker than the other activitities, in the untreated original extract, it can hardly be decided, whether it was totally inactivated at this pH.

Treatment with alkali was also undertaken almost on the same way. The activities were decreased at pH over 10. The degree of inactivation was almost the same among the three activities.

(4) Differentiation by heat inactivation.

The original extract was divided into six portions and five of them were heated for 30 minutes at 50°, 60°, 70°, 75°, and 80°C, respectively. After centrifugation,

Table IV

Heat Inactivation of Pig Kidney Extract.

Temperature of heating, °C.	Substrate	Acidity increase in 5 ml. digestion mixture (ml. 0.1 N NaOH)					
		Time of incubation in hours.					
		0.5	1	2	6	24	48
Not heated	G-G	0.90	1.20	1.50	—	—	—
	AcG	0.63	1.10	1.58	—	—	—
	BzG	—	—	|	0.23	0.93	1.71
50	G-G	0.55	0.75	0.95	—	—	—
	AcG	0.86	1.31	1.58	—	—	—
	BzG	—	—	—	0.35	1.05	1.55
60	G-G	0.25	0.50	0.84	—	—	—
	AcG	0.75	1.22	1.50	—	—	—
	BzG	—	—	—	0.20	0.90	1.44
70	G-G	0.13	0.18	0.27	—	—	—
	AcG	0.50	0.92	1.32	—	—	—
	BzG	—	—	—	0.11	0.42	0.86
75	G-G	0.00	0.04	0.04	—	—	—
	AcG	0.05	0.12	0.31	—	—	—
	BzG	—	—	—	0.02	0.06	0.26
80	G-G	0.00	0.00	0.00	—	—	—
	AcG	0.00	0.00	0.02	—	—	—
	BzG	—	—	—	0.00	0.00	0.00

G-G : Diglycine. AcG : Acetylglycine. BzG : Benzoylglycine.

the supernatants were measured of their enzymatic activities.

The effect of heating can be seen from the results shown in Table IV. Contrary to the case of acid inactivation, the dipeptidase activity was not most resistant to heating. From these facts it is evident that the activity against acetylglycine and that against diglycine in the kidney extract can be ascribed to dif-

ferent enzymes.

Whether the activities against acetylglycine and benzoylglycine were based on the same enzyme or on the different ones could not be decided as yet.

(5) Differentiation by purification.

If the purification of one of the enzymes is performed and the other enzyme activities can be reduced, it is finally confirmed that the different activities are based on the different enzymes.

The purification of acylase will be fully described in the next report. The following procedure was a preliminary one, by which the acylase activity per nitrogen could be enriched about 30 times, while the dipeptidase activity remained practically unchanged. Preliminary procedure of the purification was as follows: The original extract was acidified to pH 4.7 with 1 N HCl. After being centrifuged and neutralized, the clear redish supernatant was precipitated with ammonium sulfate. It was then fractionated with acetone at low temperature. The fraction obtained by making acetone concentration from 30 to 60 per cent, was used as the enzyme preparation.

In Table V are presented the results obtained with the original extract and the purified preparation. The enzymatic activity against diglycine in the form-

TABLE V

Differentiation of Enzymatic Activities of pig Kidney Extract*

Enzyme Material	Substrate	Acidity increase in 5 ml. digestion mixture (ml. 0.1 N NaOH)					
		Time of incubation in hours.					
		1/4	1/2	1	4	8	20
Original extract	G-G	0.66	1.25	1.62 (4.8)	—	—	—
	AcG	0.29	0.29	0.56 (1.0)	—	—	—
	AcGG	—	—	—	0.05	0.10	0.12
	BzG	—	—	—	0.19	0.47	0.70
	BzGG	—	—	—	0.20	0.34	0.46
Purified fraction	G-G	0.13	0.25	0.43 (3.3)	—	—	—
	AcG	1.01	1.60	1.75 (26.9)	—	—	—
	AcGG	—	—	—	0.00	0.00	0.00
	BzG	—	—	—	0.10	0.26	0.73
	BzGG	—	—	—	0.00	0.00	0.00

Values shown in parentheses are those of specific activity.

The specific activity was defined as the acidity increase per hour per nitrogen. Confer the work which will follow.

G-G : Diglycine. AcG : Acetylglycine. AcG G : Acetyldiglycine.

BzG : Benzoylglycine. BzGG : Benzoyldiglycine.

er was larger than that against acetyglycine, while in the latter the relationship was reversed by the purification and the acylase became most active. Thus the two activities were finally distinguished as based on two different enzymes.

As to the activities against acetyldiglycine, benzoyldiglycine and benzoylglycine, the former two which can be referred to as carboxypeptidase, were reduced to tally, while the latter remained to some extent. Hence it is clear that the acylase activity and the carboxypeptidase activity actually rest on the different enzymes: acylase and carboxypeptidase, respectively. The activity against benzoylglycine can be attributed to hippuricase. The hippuricase activity remained in the purified preparation was somewhat reduced, compared with the acylase activity. But the former was contained from the first, not much in the original extract. Hence it is dubious whether the actual reduction had occurred or not. The structures of benzoylglycine and acetylglycine are similar in that only the acyl groups are different. So that the differentiation of the hippuricase from the acylase activity is interesting. But unfortunately it could not be clarified by the present experiment.

(6) Effect of various substances on the acylase activity.

(a) At first the effect of various metal ions on the acylase was studied. As can be seen from Table VI, it was inhibited by Cu^{++}, Ag^{+}, and Hg^{++} ions perfectly,

TABLE VI

Effect of Inorganic Salts on the Pig Kidney Acylase.

Substance added.	Hydrolysis of acetylglycine in percentage, after incubation of 20 hours.	Substance added.	Hydrolysis of acetylglycine in Percentage, after incubation of 20 hours.
$CuSO_4$	0	$AgNO_3$	0
$NiSO_4$	79	$MgCl_2$	82
$MnSO_4$	79	$Pb(CH_3COO)_2$	102
$CoCl_2$	92	$SnCl_2$	90
$FeSO_4$	93	$SnCl_4$	90
$Fe_2(SO_4)_3$	66	$Al_2(SO_4)_3$	102
$HgCl_2$	0	$CaCl_2$	90
HgCl	102	Na_2SO_4	98

As to the experimental conditions, cf. text.

which would be ascribed to the denaturation of the enzyme protein by the heavy metal ions used.

(b) In Table VII are presented the results obtained with amino acids. Obvious inhibition was observed only with cystein. The inhibition by cystein may be specific, since glycine, which would have caused competitive inhibition as the component of the substrate, benzoylglycine, did not inhibit the enzyme in the concentration used.

(c) The cathepsin can be activated by cystein and hydrogen sulfide and the activation was accribed to the peptidase contained in the cathepsin preparation

TABLE VII

Effect of Amino Acids on the Pig Kidney Acylase

Amino acid	Hydrolysis of acetylglycine in percentage, after incubation of 3 hours.
Glycine	115
L-Alanine	95
D, L-Valine	101
L-Leucine	90
L-Cysein	14
D, L-Methionine	81
D, L-Aspartic acid	110
L-Glutamic acid	110
L-Ornithine	97
D, L-Lysine	112
L-Histidine	74

As to the experimental conditions, cf. text.

(12). Thus it is interesting to test the effect of substances which can affect the cathepsin. From Table VIII it can be seen that H_2S, phenyl hydrazine and aniline had no effect on the acylase.

TABLE VIII

Effect of Hydrogen Sulfide, Phenylhydrazine and Anilin on the Pig Kidney Acylase.

Substance added	Hydrolysis of acetylglycine in percentage, after incubation for 20 hrs.
H_2S*)	102
Phenylhydrazine	96
Aniline	103

*) 5ml. of H_2S saturated water were added to 25ml. of digestion mixture.

As to the experimental conditions, cf. text.

(d) Further the effect of halogenacetic acid, was examined, since they inhibited the cathepsin readily. From the results shown in Table IX some inhibition of acylase by chloro, bromo and iodoacetic acids can be seen. The degree of inhibition by the three acids were almost the same. But it was not marked, as in the case of cathepsin.

The enzyme solution used for this experiment was not the original extract, but a partially purified acylase prepartion as described above. The digestion mixture for the measurement of enzyme activity consisted of 0.5 ml. of enzyme solution, 0.5ml. of 0.066 M phosphate buffer, 0.5ml. of the solution of the sub-

stance to be tested and 1 ml. of either 0.2 M neutralized substrate solution or water.

TABLE IX

Effect of Halogenacetic Acid on Partially Purified Preparation of Kidney Acylase.

Concentration of acid added	Hydrolysis of acetylglycine in percentage, after incubation for 20 hrs.		
	Chloroacetic A.	Bromoacetic A.	Iodoacetic A.
0.02 M	—	—	37
0.01 M	73	63	56
0.005 M	92	86	74
0.0025 M	107	—	—

As to the experimental conditions, cf. text.

(e) As *Greenstein* et al(13)reported that the acylase was activated by Co^{++} ion, the effect of it was examined with the partially purified preparation. As can be seen from the results presented in Table X, the inhibition by Cu^{++} -ion was readily observed, but the effect of Co^{++} -ion was not notable.

TABLE X

Effect of $CuSO_4$ and $CoCl_2$ on the partially Purified Preparation of Kidney Acylase.

Concentration of added substance	Hydrolysis of acetylglycine in percentage after incubation for 30min.	
	$CoCl_2$	$CuSO_4$
0.01 M	68	19
0.005 M	88	33
0.0025 M	110	—

As to experimental conditions, cf. tex.

Greenstein et al studied also the specificity of acylases. Purifying the enzymes partially, they differentiated them into acylase I and II. In the present work the specificity of acylase was not taken into consideration and only one substrate, acetylglycine, was used.

Against the acid treatment, the dipeptidase was more resistant than the acylase and to the heat inactivation, the acylase. Hence, for the purpose of the purification of acylase, the heat inactivation will offer a favourable methed. The fractionation by high speed centrifugation made an effective differentiation of the two enzymes. Especially it seemed to be favourable for the dipeptidase, since the acylase was contained very little in the sediment, compared with the former. On the contrary, the dipeptidase was not only contained in the sediment, but also dissolved in the supernatant. So that the acylase could not be separated from it. Therefore the high speed centrifugation may be dispensable for the purification of the acylase.

SUMMARY

Optimal pH for the hydrolysis of acetylglycine and diglycine by kidney extract were 7.0 and 8.0, respectively. The two enzymatic activities could be distinguished from each other by fractional high speed centrifugation. Thus they could be ascribed to the different enzymes: acylase and dipeptidase.

By acid treatment the former was most inactivated and by heating, the latter.

By a partial purification of acylase, it was enriched about 30 times, while the dipeptidase remained unchanged.

Effects of various substances on the kidney acylase were examined.

Grateful acknowledgements are due to Prof. S. Utzino of the Kyoto University and Prof. S. Nakamura of the Yamaguchi Medical School for their kind advise in this study. This work is in part supported by a grant from the Ministry of Education.

LITERATURE

1) ABDERHALDEN, E.: *Fermentforschung*, **12**, 225,1931.
2) KIMURA, H.: *J. Biochem.*, **10**, 225, 1929.
3) MAYEDA, H.: *Acta School. Med. Univ. Kioto*, **18**, 205, 1936.
4) UTZINO, S. AND NAKAYAMA, M.: *Enzymologia*, **8**, 280, 1940.
5) AKIZUKI, H.: *J. Biochem.*, **25**, 43, 1937.
6) MATSUI,: *J. Biochem.*, **33**, 183, 1941.
7) MASATAKA, M.: *J. Biochem.*, **33**, 147, 1941.
8) FODOR, P. J., AND GREENSTEIN, J. P.: *J. Biol. Chem.*, **189**, 549, 1949.
9) HERBST, R. M., AND SHEMIN. D,: *Org. Synthesis*, coll. **2**, 11, 1943.
10) FREUDENBERG, K., EICHEL, H., LEUTERT, F.: *Ber. dtsch. chem. Ges.*, **65**, 1191,
11) FISCHER, E.: *Ber. dtsch. chem. Ges.*, **38**, 609, 1905.
12) FRUTON, J. S. AND BERGMANN, M.: J. Biol. Chem. **130**, 19, 1939.
13) BIRNBAUM, S. M., LEVINTOW, L., KINGSLEY, R.B. AND GREENTSEIN, J. P.: *J. Biol. Chem.*, **194**, 455, 1952.