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Studies on Continuous Enzyme Reactions

Part VI. Enzymatic Properties of the DEAE-Sephadex-Aminoacylase Complex

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The enzymatic properties of the water-insoluble aminoacylase prepared by linking mold aminoacylase (EC 3.5.1.14) to DEAE-Sephadex were studied and compared with those of the native aminoacylase.

Optimum pH values for hydrolysis of several substrates by the DEAE-Sephadex-aminoacylase complex (DSA-complex) shifted about 0.5~1.5 pH units more to the acid side than those by the native enzyme. On the effects of metal ions and inhibitors, substrate specificity, optical specificity and kinetic constants, no marked difference was observed between the native enzyme and the DSA-complex. Heat stability, optimum temperature and resistance towards proteases were increased by conversion from the native form to the insoluble enzyme. It was also observed that the DSA-complex was activated by urea.

In previous papers of this series,[1~5] the authors revealed that the continuous optical resolution of acyl-DL-amino acids was possible by using the column packed with the water-insoluble DCA-complex* or DSA-complex,* and further investigated the enzymatic properties of DCA-complex.

In this presentation, the enzymatic properties of the water-insoluble DSA-complex were investigated and compared with those of the native aminoacylase.

* Abbreviation: DCA-complex, DEAE-cellulose-aminoacylase complex; DSA-complex, DEAE-Sephadex-aminoacylase complex.

1) T. Tosa, T. Mori, N. Fuse and I. Chibata, *Enzymologia*, **31**, 214 (1966).
2) T. Tosa, T. Mori, N. Fuse and I. Chibata, *ibid.*, **31**, 225 (1966).
3) T. Tosa, T. Mori, N. Fuse and I. Chibata, *Biotech. Bioeng.*, **9**, 603 (1967).
4) T. Tosa, T. Mori, N. Fuse and I. Chibata, *Agr. Biol. Chem.*, **33**, 1047 (1969).
5) T. Tosa, T. Mori, N. Fuse and I. Chibata, *Enzymologia*, **32**, 153 (1967).

MATERIALS AND METHODS

Materials. DEAE-Sephadex A-25 of 3.5±0.5 meq/g (bead type, 40~120 μ) was obtained from Pharmacia (Uppsala, Sweden). The acylamino acids employed were prepared by the method described in the literature.[6] As the native aminoacylase, aqueous extract of wheat bran culture of *Aspergillus oryzae* No. 9 prepared by the method described in the previous paper[1] was used. Activity of the extract determined by standard enzyme assay was 20 μmoles/hr/ml of extract.

DCA-complex was prepared by the method described in the previous paper.[5]

Preparation of DSA-complex. At 5°C, 18.5 g of DEAE-Sephadex A-25 (hydroxy form) suspended in 100 ml of distilled water was stirred with 1670 ml of native aminoacylase solution (33,400 μmoles/hr) for 5 hr and kept standing overnight. After filtration, 1000 ml of distilled water was added to the precipitate, stirred

6) J. P. Greenstein and M. Winitz, "Chemistry of Amino Acids", Vol. 3, John Wiley & Sons, Inc., New York, 1961.

for 1 hr, and filtered. This washing process was repeated once more. For further washing, 1000 ml of 0.2 M sodium-acetate were added to the insoluble complex, stirred for 1 hr, and filtered. The precipitate was washed with 1000 ml of distilled water as described above. These washing procedures were carried out at 25°C. No aminoacylase activity was detected in the washing solution. The resulted precipitate was suspended in 500 ml of distilled water and lyophillized. By this procedure, 19.6 g of DSA-complex was obtained. Total activity of the preparation was 15,700 μmoles/hr under standard conditions for enzyme assay and 41,600 μmoles/hr at optimum temperature (72°C).

Standard enzyme assay of native aminoacylase. Unless otherwise noted, standard enzyme reaction of native aminoacylase was carried out by the method previously described.[5]

Standard enzyme reaction of DSA-complex. Unless otherwise noted, standard enzyme reaction of DSA-complex was carried out as follows: a reaction mixture of 10 ml of M/15 veronal buffer (pH 7.0), 10 ml of M/15 acetyl-DL-methionine (pH 7.0) containing 1×10^{-3}M Co^{2+} and 100 mg of DSA-complex was incubated for 30 min at 37°C with shaking. After the reaction, the aminoacylase complex in the reaction mixture was filtered off, and liberated L-methionine in the filtrate was measured by the ninhydrin colorimetric method using the Technicon Auto-Analyzer system.[7] The enzyme activity of the aminoacylase complex was expressed in micromoles of L-methionine liberated per hour. The effects of enzyme concentration and incubation period on the reaction rate are shown in Fig. 1 and Fig. 2, respectively.

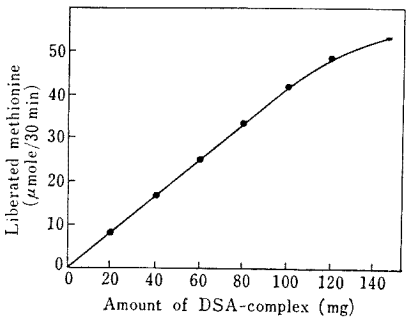

FIG. 1. Effect of Concentration of DSA-Complex on the Reaction Rate.

The enzyme assay was carried out under standard conditions with varied amounts of enzyme.

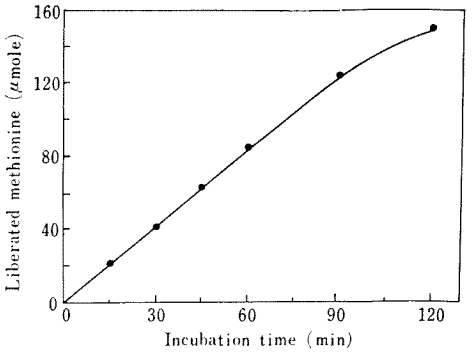

FIG. 2. Effect of Incubation Period on the Reaction Rate.

The enzyme assay was similar to the standard conditions except varying incubation time.

RESULTS

Effect of pH

In a previous paper,[5] the authors revealed that the optimum pH values of DCA-complex towards several α-N-acetylamino acids and chloroacetyl-DL-alanine apparently shift about 0.5~1.0 pH unit more towards the acid side than those of the native aminoacylase. Then, the effect of pH on the hydrolytic rate by DSA-complex was studied. Comparison of the results with native aminoacylase is summarized in Table I.

The optimum pH values of DSA-complex for α-N-acetyl derivatives of methionine, phenylalanine, tryptophan and valine shifted about 0.5~1.5 pH units more towards the acid side than those of the native aminoacylase. However, no shift of optimum pH values was observed for the hydrolysis of α-N-chloroacetyl derivatives of phenylalanine, tryptophan and tyrosine. These results are quite the same as in the case of DCA-complex.

7) N. G. Cadavid and A. C. Paladini, *Anal. Biochem.*, **9**, 170 (1964).

TABLE I. OPTIMUM pH FOR HYDROLYSIS OF ACYLAMINO ACIDS

Acylamino acids	Optimum pH	
	Native aminoacylase	DSA-complex
Acetyl-DL-methionine	7.5	7.0
Acetyl-DL-phenylalanine	7.5	6.0
Acetyl-DL-tryptophan	7.5	6.0
Acetyl-DL-valine	7.0	6.0
Chloroacetyl-DL-phenylalanine	6.0	6.0
Chloroacetyl-DL-tryptophan	6.0	6.0
Chloroacetyl-L-tyrosine	6.0	6.0

The enzyme assay was carried out under standard conditions except for the substrates and buffers employed. At pH=4.0~6.0, pH=5.5~8.0, and pH=7.0~9.5, M/15 acetate buffer, M/15 phosphate buffer, and M/15 veronal buffer were employed, respectively.

Effect of metal ions and inhibitors

The effects of metal ions and inhibitors on the hydrolysis of acetyl-DL-methionine were investigated. The results are shown in Table II.

Compared with the native aminoacylase, the inhibitory effect of Fe^{2+}, Hg^{2+} and *p*-chloromercuribenzoate on the DSA-complex was higher. The DSA-complex was also specifically activated by Co^{2+} as in the cases of the DCA-complex and the native enzyme.

TABLE II. EFFECT OF METAL IONS AND INHIBITORS ON THE ENZYME ACTIVITY

Metal ions and inhibitors	Relative activity*	
	Native aminoacylase	DSA-complex
None	100	100
Ba^{2+}	105	80
Ca^{2+}	96	76
Cd^{2+}	9	12
Co^{2+}	146	141
Fe^{2+}	101	59
Hg^{2+}	77	7
Monoiodoacetate	88	80
p-Chloromercuribenzoate	98	46
EDTA	19	16
NaCN	101	86

The enzyme assay was carried out under standard conditions modified by the addition of metal ions or inhibitors in place of Co^{2+}. Metal chlorides were employed as metal ions except for Mohr's salt. Concentration of metal ions and inhibitors in the reaction mixtures was 1×10^{-3}M.

* The activity without metal ions or inhibitors was taken as control (100).

Effect of urea

The effect of urea on the activity of three preparations, native aminoacylase, DCA-complex and DSA-complex was investigated. In this experiments, the enzyme preparations were incubated for 1 hr at 25°C at pH 6~8 in 6 M urea, and the enzyme activity was assayed both in the presence of 2 M urea and after the removal of urea.

As shown in Table III, the enzyme activity

TABLE III. EFFECT OF UREA ON THE ENZYME ACTIVITY

Enzyme preparations	Enzyme activity (Per cent of initial activity)	
	Under presence of urea	After removal of urea
Native aminoacylase	17	56
DSA-complex	130	92
DCA-complex	121	63

In the case of the native aminoacylase, a mixture of 1 ml of native aminoacylase dissolved in M/10 veronal buffer (pH 7.0) and 2 ml of 9 M urea solution was incubated for 1 hr at 25°C, and 6 ml of M/20 acetyl-DL-methionine (pH 7.0, containing 7.5×10^{-4} M Co^{2+}) was added. The mixture was incubated under standard enzyme assay conditions.

In the case of the aminoacylase complexes, a mixture of 5 ml of M/10 veronal buffer (pH 7.0), 10 ml of 9 M urea solution and 100 mg of DCA-complex or 40 mg of DSA-complex was incubated with shaking for 1 hr at 25°C, and 30 ml of M/20 acetyl-DL-methionine (pH 7.0, containing 7.5×10^{-4}M Co^{2+}) was added. The mixture was incubated under standard enzyme assay conditions.

In both cases, liberated methionine was determined, and the values of initial activity were taken as control (100%).

In the removal experiments of urea, it was removed from the urea-treated media by dialysis (in the case of native aminoacylase) or filtration (in the cases of aminoacylase complexes), and enzyme activities of dialysate (in the former case) and of precipitate (in the latter case) were measured under conditions of the standard enzyme assay.

of the native aminoacylase was inhibited by urea and partly recovered by the removal of urea. It is interesting to note that both insoluble enzyme preparations were activated by urea, and the activation was not observed after the removal of urea.

Effect of proteases

The effect of proteases on the native aminoacylase and the insoluble aminoacylases was investigated, and the results are shown in Table IV

TABLE IV. EFFECT OF PROTEASES ON THE ENZYME ACTIVITY

Proteases	Per cent of remaining activity		
	Native aminoacylase	DSA-complex	DCA-complex
Pronase-P*	68	88	53
Trypsin**	23	87	33

* Preparation from Kaken Chemical Co.

** Preparation from N. B. C.

In the case of the native aminoacylase, a mixture of 0.5 ml of native aminoacylase, 1.0 ml of M/10 phosphate buffer (pH 7.0) and 0.5 ml of 1% protease was incubated for 24 hr at 37°C, and 1 ml of M/10 acetyl-DL-methionine (pH 7.0, containing 1.5×10^{-3}M Co^{2+}) was added for enzyme assay. In the case of the aminoacylase complexes, a mixture of 50 mg of DSA-complex or 100 mg of DCA-complex, 10 ml of M/15 phosphate buffer (pH 7.0) and 5 ml of 0.75% protease was incubated with shaking for 24 hr at 37°C, and 15 ml of M/15 acetyl-DL-methionine (pH 7.0, containing 1×10^{-3}M Co^{2+}) was added for enzyme assay. These reaction mixtures were incubated under conditions of the standard enzyme assay. Liberated methionine was determined, and the values obtained in the cases of the reaction mixtures containing no proteases were taken as control (100%).

The results indicate that activity of DCA-complex was lowered by the treatment with proteases as well as in the case of native aminoacylase. However, the activity of DSA-complex was not so decreased as other two preparations; that is, DSA-complex is the most unsusceptible towards the action of proteases.

Stability in storage

To investigate the stability of DSA-complex, it was stored for period of 5 months at 5°C and 25°C under the conditions of dry state and suspension in substrate solution or in distilled water, and the remaining activity was assayed. The results are shown in Fig. 3.

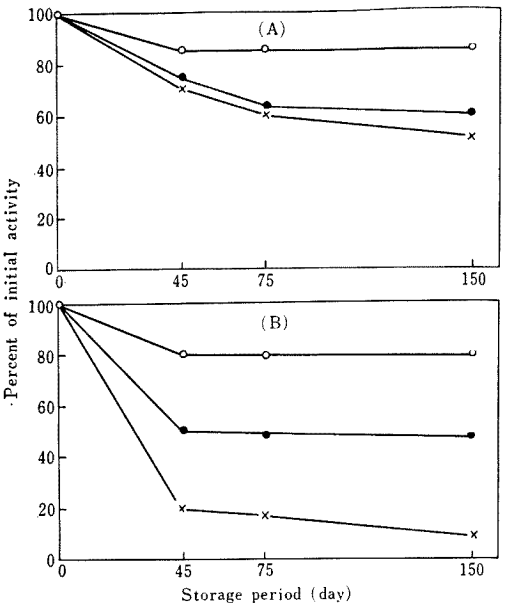

FIG. 3. Stability of DSA-Complex in Storage.

DSA-complex was stored for 45~150 days at 5°C (Fig. A) and 25°C (Fig. B) under the conditions of dry (○—○), suspension in 0.2 M acetyl-DL-methionine solution (pH 7.0, containing 5×10^{-4}M Co^{2+}) (●—●), and in distilled water (×—×). After storage, the enzyme activity of the complex was measured under standard conditions.

Under the dry condition, over 80% of the enzyme activity remained after being kept for 5 months at either temperature. Between the suspensions in substrate solution and in distilled water, the former gave better result.

Heat stability

The effect of temperature on the stability of the aminoacylase complexes and the native aminoacylase was investigated by incubating these preparations at 65~80°C for 15 min, and the results are shown in Fig. 4.

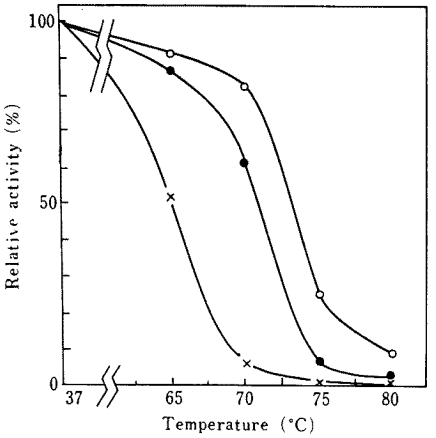

FIG. 4. Heat Stability.

In the case of the native aminoacylase (×—×), a mixture of 1 ml of M/10 veronal buffer (pH 7.0) and 1 ml of native aminoacylase was incubated for 15 min at the specified temperature, rapidly cooled, and 1 ml of M/10 acetyl-DL-methionine (pH 7.0) containing 1.5×10^{-3}M Co^{2+} was added for enzyme assay. In the case of the aminoacylase complexes, a mixture of 10 ml of M/15 veronal buffer (pH 7.0) and 100 mg of DCA-complex (●—●) or 40 mg of DSA-complex (○—○) was incubated with shaking for 15 min at the specified temperature, rapidly cooled, and 10 ml of M/15 acetyl-DL-methionine (pH 7.0) containing 1×10^{-3}M Co^{2+} was added. These reaction mixtures were incubated under conditions of the standard enzyme assay. Liberated methionine was determined, and the values obtained at the pre-incubation temperature of 37°C were taken as 100%.

The Figure shows that DSA-complex was most stable among the aminoacylase preparations tested.

Effect of temperature on the reaction rate

The effect of temperature on the reaction was investigated, and the results are shown in Fig. 5.

The Figure indicates that the optimum temperature for DSA-complex was the highest, *i.e.*, 72°C, among three preparations tested.

For the estimation of the apparent activation energy of these preparations on acetyl-DL-methionine, the results shown in Fig. 5 were

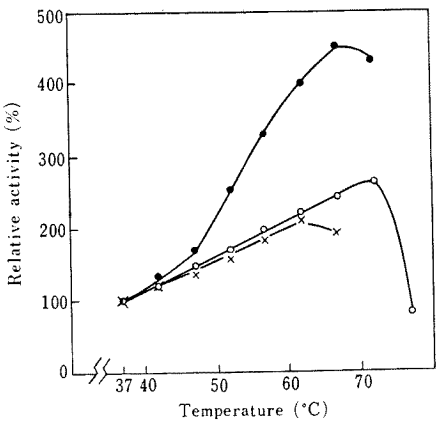

FIG. 5. Effect of Temperature on the Reaction Rate.

The enzyme assay was carried out under standard conditions modified by varying the incubation temperature. The reaction rates obtained at 37°C were taken as 100%.

×—× Native aminoacylase
●—● DCA-complex
○—○ DSA-complex

plotted by the method of Arrhenius. Thus, it was found that the apparent activation energies of DCA-complex, DSA-complex and native aminoacylase were calculated to be 11,100 cal/mol., 7,000 cal/mol. and 6,700 cal/mol., respectively.

Substrate specificity

In order to clarify the specificity of DSA-complex, susceptibility of several acylamino acids was investigated. The results, obtained under the presence of Co^{2+} at optimum pH of respective acylamino acids, are shown in Table V.

From the Table it becomes clear that the DSA-complex has also optical specificity because acetyl-D-methionine was not hydrolyzed by the enzyme, and that the tendencies of the hydrolytic activities of both enzymes towards most substrates are the same.

Kinetic constants for several substrates

The effect of substrate concentration on the

TABLE V. SUBSTRATE SPECIFICITY

Substrates	Relative activity	
	Native aminoacylase	DSA-complex
Acetyl-DL-methionine	100	100
Acetyl-D-methionine	0	0
Acetyl-DL-phenylalanine	129	94
Acetyl-DL-tryptophan	93	109
Acetyl-DL-valine	39	67
Chloroacetyl-DL-phenylalanine	106	152
Chloroacetyl-DL-tryptophan	54	59
Chloroacetyl-L-tyrosine	96	89

The enzyme reaction was carried out under standard conditions except for the substrate employed. Concentration of substrate in the reaction mixture was M/30 for racemic compounds and M/60 for optically active compounds. The liberated amino acids was determined by the ninhydrin colorimetric method using the Technicon Auto-Analyzer system. The activity towards acetyl-DL-methionine was taken as control (100) for the both enzyme preparations.

TABLE VI. KINETIC CONSTANTS FOR SEVERAL SUBSTRATES

Substrates	Km (mM)		V_{max} (μmole/hr)	
	Native amino-acylase	DSA-complex	Native amino-acylase	DSA-complex
Acetyl-DL-methionine	5.7	8.7	1.52	3.33
Acetyl-DL-phenylalanine	3.3	6.8	1.85	2.94
Acetyl-DL-tryptophan	8.1	4.9	0.91	2.86
Acetyl-DL-valine	41	18	1.66	2.50

Reaction rates were measured under standard enzyme conditions except for the substrates employed and their concentration. Apparent Michaelis constant (Km) and maximum velocity (V_{max}) for both enzyme preparations were determined from Lineweaver-Burk's plots.

enzyme activity was investigated, and the results obtained were plotted by the method of Lineweaver and Burk for the estimation of the apparent Michaelis constants and maximal velocities. The results are shown in Table VI.

As shown in Table VI, no marked difference of the kinetic constants was observed between these two enzyme preparations. The results are the same as the case of DCA-complex previously reported.[5]

DISCUSSION

Although a number of reports have been published on the enzymatic properties of the water-insoluble enzymes, little is known about a water-insoluble aminoacylase. Accordingly, the enzymatic properties of the DCA-complex —a kind of "modified enzyme"—were investigated, and interesting results as reported in the foregoing paper[5] were obtained. In this presentation, the enzymatic properties of the DSA-complex were investigated. The DSA-complex has higher enzymatic activity than the DCA-complex and is advantageous over the DCA-complex for continuous optical resolution of acyl-DL-amino acids.

The optimum pH values of DSA-complex towards several substrates were found to shift more towards the acid side as well as in the case of DCA-complex in comparison to native enzymes. As discussed in detailed in the Levin *et al.*[8] and Goldstein *et al.*,[9] this shift in the cases of both insoluble enzymes may also be explained by the redistribution of hydrogen ions between the positively charged enzyme carriers (DEAE-cellulose or DEAE-Sephadex) and the surrounding aquous medium.

Of the tested properties, significant difference between the native enzyme and the DSA-complex was observed on the effect of temperature, such as heat stability and optimum temperature. These results suggest as in the case of DCA-complex that some conformation change in the protein structure of the DSA-complex occurs. However, there was no significant difference between kinetic constants of both enzyme preparations, indicating that conversion to insoluble form caused no marked

8) Y. Levin, M. Pecht, L. Goldstein and E. Katchalsky, *Biochemistry*, **3**, 1905 (1964).
9) L. Goldstein, Y. Levin and E. Katchalsky, *ibid.*, **3**, 1913 (1964).

conformation change in the protein structure.

Among the native enzyme and both insoluble enzymes, DSA-complex was most unsusceptible towards the action of proteases. This results due to changes of the susceptibility of these aminoacylase preparations towards the action of proteases by the fixation of the native enzyme on a water-insoluble carrier. This property of DSA-complex is considered to be advantageous for industrial use, because the complex is resistant to deterioration caused by proteases of microorganisms contaminated during long time operation.

It is very interesting that DSA-complex and DCA-complex were activated by urea, and seems to be a first time that a water-insoluble enzyme is activated by a protein denaturing agent. This activation is considered to occur by the conversion of rigid structure of the insoluble enzymes into flexible structure by urea. In succession to this report, detailed studies on this activation by urea, guanidine hydrochloride and organic solvents as protein denaturing agents will be reported elsewhere.

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