Aminoacylase from *Aspergillus oryzae*. Comparison with the Pig Kidney Enzyme

Ingrid Gentzen, Hans-G. Löffler, and Friedh. Schneider

Physiologisch-Chemisches Institut der Universität Marburg, Lahnberge, D-3550 Marburg

Z. Naturforsch. **35 c,** 544–550 (1980); received February 21, 1980

Aspergillus Aminoacylase, Purification, Properties

Aminoacylase (EC 3.5.1.14) from *Aspergillus oryzae* was purified from a commercially available crude material by heat treatment, precipitation by polyethyleneimine and ammoniumsulfate, gel chromatography and preparative disc-gel-electrophoresis. The purified product was homogenous as judged by polyacrylamide gel electrophoresis.

SDS-gel electrophoresis, polyacrylamide-gel-gradient electrophoresis, gel chromatography and amino acid analysis demonstrated the enzyme to be composed of two subunits with M_r of 36 600.

The kinetic properties of the enzyme were studied with chloroacetyl derivatives of alanine, phenylalanine, methionine, leucine, norleucine and tryptophan. The pH optimum of the acylase activity with chloroacetyl-alanine as substrate is at pH 8.5. Acyl derivatives of hydrophobic amino acids are preferred substrates. The enzyme has no dipeptidase activity.

Aminoacylase is not inhibited by SH-blocking agents and no SH-groups could be detected with Ellman's reagent in the native and denatured enzyme. The enzyme activity is insensitive to phenylmethylsulfonyl fluoride and N-α-*p*-tosyl-L-lysine chloromethyl ketone.

The microbial acylase is a zinc metallo enzyme. Metal chelating agents are strong inhibitors; it is further inhibited by Cd^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+} and activated by Co^{2+}.

The properties of pig kidney and *Aspergillus* acylase are compared.

The enzyme aminoacylase (EC 3.5.1.14) is widely distributed in nature; it is found in many mammalian tissues [1, 2] and in microorganisms [3–5]. Its utilisation for the resolution of racemic amino acids by way of their acylderivatives is well known [6]. The physiological function of aminoacylase is not quite clear. It seems likely that the enzyme functions in the catabolism of N-acetylated proteins, which are abundant in mammalian cells [7]. The sequence of events leading to removal of the acetyl blocking groups appears to involve proteases releasing an N-terminal acetylated peptide from the protein, further acylpeptide hydrolase [8–10] which catalyses the release of the N-acetyl amino acid from the peptide, and finally aminoacylase which hydrolyses the acetyl amino acid to yield acetate and the N-terminal amino acid.

While the chemical and catalytic properties of aminoacylase from pig kidney have been thoroughly investigated [6, 11–16, 30], little is known about microbial aminoacylases. The present paper therefore describes some molecular and kinetic properties of aminoacylase from *Aspergillus oryzae* and presents a comparison of the mammalian and microbial enzyme.

Materials and Methods

Aminoacylase from *Aspergillus oryzae* was a gift from Boehringer, Mannheim. N-chloroacetyl derivatives of L-alanine, L-leucine, L-methionine, DL-norleucine, L-phenylalanine and L-tryptophan were synthesized as described by Greenstein *et al.* [6]. N-dichloroacetylglycine, N-dichloroacetyl-L-leucine were synthesized as described by Ronwin [17], N-dichloroacetyl-L-alanine by Krebs and Schumacher [18], N-dichloroacetyl-DL-phenylalanine by Kameda *et al.* [19] and N-chloroacetyl-γ-L-glutamyl-*p*-aminobenzoic acid (Cl-Ac-Glu-PABA) by Szewczuk and Wellman-Bednawska [20]. N-α-acetyl derivatives of L-glutamine, L-glutamic acid, L-alanine, L-lysine and L-aspartic acid were from Sigma (Munich). Reference proteins for molecular weight determinations were from Serva, Heidelberg, Boehringer, Mannheim or Sigma, Munich. Asparaginase and polyethylene imine (Polymin P) were a gift from Bayer, Wuppertal. Sephadex G-150 and Sepharose 6-B-Cl were from Pharmacia, Frankfurt. Buffer substances and

Enzymes: Aminoacylase, N-acylamino-acid amidohydrolase (EC 3.5.1.14); asparaginase, L-asparaginase amidohydrolase (EC 3.5.1.1); alkaline phosphatase, orthophosphoric monoester phosphohydrolase (EC 3.1.3.1); β-galactosidase, β-D-galactoside galactohydrolase (EC 3.2.1.23).

Reprint requests to Dr. Fr. Schneider.

0341-0382/80/0700-0544 $ 01.00/0

other chemicals were p. a. grade from Merck, Darmstadt. All reagents for chemical modification of proteins were from Serva, Heidelberg, except N-ethylmaleimide and N-α-*p*-tosyl-L-lysine chloromethyl ketone HCl, which were from Merck, Darmstadt.

Kinetic measurements

Kinetic Parameters were measured in a 0.1 M acetate/phosphate/borate buffer pH 7.0, or in a 0.03 M phosphate buffer pH 8.0 at 40 °C with a Zeiss PMQ II spectrophotometer. Hydrolysis of N-chloroacetyl tryptophan was followed at 295 nm, of acetyl-derivatives of amino acids at 228 nm and of all other N-chloro- and N-dichloroacetylderivatives at 238 nm. Activity measurements with Cl-Ac-Glu-PABA were carried out as described by Szewczuk and Wellman-Bednawska [20].

Zinc analysis of the purified enzyme

The enzyme was dialyzed against a 0.01 M ammonium-acetate buffer pH 7.0, in the presence of the complexing resin Chelex 100 (Biorad Munich), which was kept in a separate dialysis bag. The buffer was deprived of metal ions by extractions with dithizone solved in carbontetrachloride. The protein concentration was determined via amino acid analysis. Zinc analysis was performed by atomic absorption spectrophotometry with a Perkin-Elmer 373 photometer equipped with a burner nebulizer assembly 057-0705 with acetylen/air flame.

Immunological methods: Antiserum against the acylase was raised in rabbits by repeated subcutaneous injection of 25–30 µg acylase antigen per kg body weight. Injections were administered weekly; the first three contained the antigen in an emulsion with Freund's complete adjuvant, in further injections incomplete adjuvant was used.

Ouchterlony double-diffusion experiments were performed according to published procedures [32].

Purification of the enzyme

Starting material for the purification of the enzyme was a commercially available product from "Amano" Nagoya Japan with a spec. act. of 0.65 U/mg protein using N-chloroacetylalanine as substrate. 10 g of the powder were dissolved in 100 ml 0.03 M potassium phosphate buffer pH 7.3. Insoluble material was removed by centrifugation and the proteolytic activity was reduced by heating the solution for 3 min to 65 °C. After dialysis against a 15 and 10 mM phosphate buffer, 100 ml of the cold solution were mixed with 31 ml of a 5% (W/W) solution of Polymin P, pH 7.3 within 40 min. The enzymatically active darkbrown slime was collected by centrifugation, resuspended with the aid of seasand and dissolved in 100 ml 60 mM phosphate buffer pH 7.3. To separate the protein from Polymin P, the enzyme was precipitated from the supernatant with ammonium sulfate (80% saturation). The precipitate was dissolved in sodium phosphate buffer and passed through a Sephadex G-150 column (4 × 100 cm) equilibrated with the same buffer. The last step of the purification of the active fractions was a preparative disc-gel-electrophoresis (7.5% polyacrylamid gel, pH 8.9) [21]. A typical purification procedure is illustrated in Table I. The enzyme was stored at 4 °C in buffer solution.

It must be emphasized that the determination of protein concentration performed by Microbiuret method (with bovine serum albumin as standard protein) and the determination by amino acid analysis resulted in different values: 1 mg protein (Microbiuret) corresponds to 0.41 mg protein via amino acid analysis for the pure Aspergillus aminoacylase. This has to be taken into consideration calculating the molar extinction coefficient of aminoacylase, the number of zinc atoms per molecule and further the spec. activity of the pure enzyme.

For the determination of the specific molar extinction coefficient, the enzyme was dialyzed against a 0.05 M phosphate buffer pH 7.3. The extinction of the solution at 280 nm was measured and the protein content was determined by amino acid analysis $E^{1\%}_{1\,cm}$ at 280 nm was found to be 14.2.

Molecular weight determination and subunit structure

Determination of the molecular weight was performed by SDS-polyacrylamide-gel electrophoresis. Aminoacylase and reference proteins were incubated according to the method of Blobel and Dobberstein [22], gel electrophoresis was carried out as described by Laemmli [23]; a 7.5% polyacrylamide-gel was used. Molecular weight determinations were accomplished by gel-filtration on Sepharose 6-B-Cl column (50 × 1.5 cm) and polyacrylamide-gel-gradient electrophoresis according to the method of Lorentz [24].

Table. I. Purification of *Aspergillus* aminoacylase starting with a commercial preparation.

Step	Protein [mg]	Activity [U]	Spec. Act. [U/mg]	Purification	Recovery of Act. [%]
Raw material	4320	2820	0.65	–	100
Heat treatment	2351	2285	0.97	1.5	81
PEI * + ammonium sulfate precipitation	16	1370	86	132	50
Sephadex G-150 chromatography	13.2	1273	96	148	45
Preparative disc-gel-electrophoresis	3.0	392	131	201	14

* Polyethyleneimine. Protein determinations were done with the microbiuret method [29].

Amino acid analysis

A Beckman Multichrom B Analyzer was used for amino acid analyses. Samples of aminoacylase were dialyzed against water and diluted with 10 N HCl to a final concentration of 6 N. After 22 h at 110 °C the samples were evaporated at 40 °C and dissolved in application buffer. Tryptophan determinations were performed according to the method of Matsubara and Sasaki [25]. Cysteine, cystine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation [26]. To provide control for the reliability of the determinations human serum albumin or papain were analyzed under the same conditions.

Effects of metal ions amd modification reagents on the activity of the enzyme

Effects of metal ions (Zn^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Cu^{2+}) on the activity of aminoacylase were tested by the following procedure: The enzyme was incubated with the metal salt solution in 0.1 M acetate/phosphate/borate buffer pH 6.0 at 40 °C for 15 min. The activity was measured with N-chloroacetylalanine as substrate.

The effects of different modification reagents of functional groups were tested by incubating aminoacylase with 0.17 mM N-ethylmaleimide, 0.2 mM iodoacetamide, 67 µM Ellman reagent [27], 2.0 mM phenylmethylsulfonyl fluoride, 1.0 mM N-α-*p*-tosyl-L-lysine chloromethyl ketone HCl and 10^{-2} M diethylpyrocarbonate. In the case of Ellman reagent the reaction was followed spectrophotometrically at 412 nm, otherwise the remaining activity was determined with N-chloroacetylalanine as substrate. All reactions were carried out in phosphate buffer pH 8.0 except the reaction with diethylpyrocarbonate which was done at pH 6.0.

Results and Discussion

Purification, stability, molecular weight, amino acid analysis

Starting material for the purification of the enzyme was a commercially available raw product from *Aspergillus oryzae* with a spec. act. of 0.65 U/mg. Typical results of one enzyme purification are summarized in Table I. A homogenous product was obtained by ammonium sulfate and polyethylenimine precipitation, gel filtration on Sephadex G-150 and preparative disc-gel-electrophoresis. When subjected to polyacrylamide gel reelectrophoresis 2 to 26 µg purified enzyme shows a single band on protein staining with coomassie G-250 in perchloric acid [28] (Fig. 1). The microbial aminoacylase is a rather stable enzyme; in 0.03 M phosphate buffer pH 7.3 and in 0.4 M NaCl the enzyme may be stored at 4 °C for several months with negligible loss of activity.

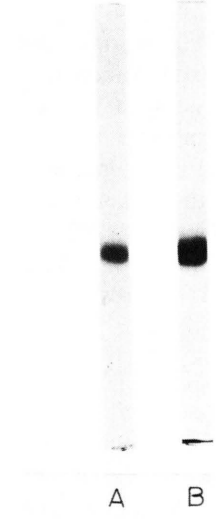

Fig. 1. Polyacrylamide gel reelectrophoresis of the purified aminoacylase. A 2.5 µg, B 6.2 µg.

Table II. Amino acid composition of the subunit of aminoacylase from *Aspergillus oryzae*.

Amino acid	Number of residues per subunit	Best integer	mol percent
Ala	32.94	33	11.22
Arg	13.13	13	4.42
Asp	34.94	35	11.90
Cys/2	1.91	2	0.68
Glu	25.23	25	8.50
Gly	23.79	24	8.16
His	7.11	7	2.38
Ile	13.06	13	4.42
Leu	23.49	23	7.82
Lys	5.43	5	1.70
Met	1.04	1	0.34
Phe	14.55	15	5.10
Pro	19.00	19	6.46
Ser	24.89	25	8.50
Thr	12.86	13	4.42
Trp	3.04	3	1.02
Tyr	5.74	6	2.04
Val	31.57	32	10.88

The amino acid analysis of the enzyme is shown in Table II. From the amino acid composition (294 amino acids) a molecular weight of 36600 is calculated per subunit. Analysis of the SH-content with Ellman reagent in the presence and absence of 8 M urea revealed no free SH-groups. After performic acid oxidation 2 cysteic acids are found, which obviously were formed from a cystine residue. The enzyme contains 6 tryptophan residues.

By gel filtration on Sepharose 6-B-Cl a M_r of about 135000 ± 10000 was found (Fig. 2). On a polyacrylamide *gel gradient* the distance of migration of the enzyme was the same as was found for the monomer of bovine serum albumin with a molecular weight of 67000 (see Fig. 3). SDS gel electrophoresis revealed the subunit structure of aminoacylase. When the distance of migration of the reference proteins and aminoacylase are plotted against the logarithms of the polypeptide chain molecular weights, the relative mobility of the main band of aminoacylase polypeptide chain corresponds to a molecular weight of 37000 (see Fig. 4). From these results it may be concluded, that the enzyme consists of two identical subunits. Obviously on gel filtration a dimer of the active enzyme is found.

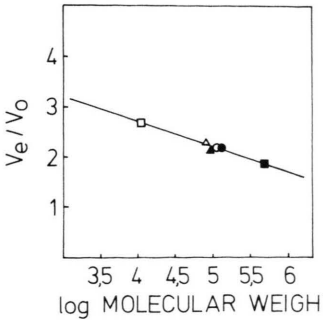

Fig. 2. Determination of the molecular weight of *Aspergillus* acylase by Sepharose 6-B-Cl chromatography. □ Cytochrom c; △ pig kidney acylase; ● asparaginase; ▲ alkaline phosphatase; ○ aminoacylase; ■ β-galactosidase. M_r of about 135000 ± 10000 was found.

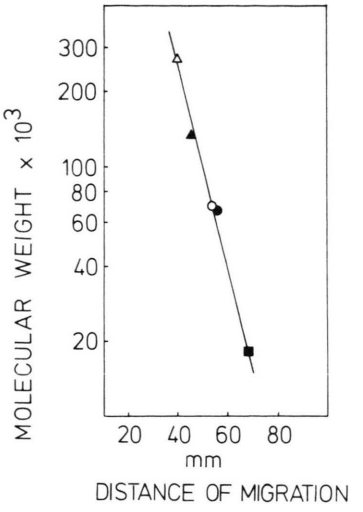

Fig. 3. Determination of the molecular weight of *Aspergillus* acylase by polyacrylamide gel gradient electrophoresis. △ Bovine serum albumin (tetramer); ○ aminoacylase; ▲ bovine serum albumin (dimer); ■ myoglobin; ● bovine serum albumin (monomer). M_r of 67000 was estimated for aminoacylase.

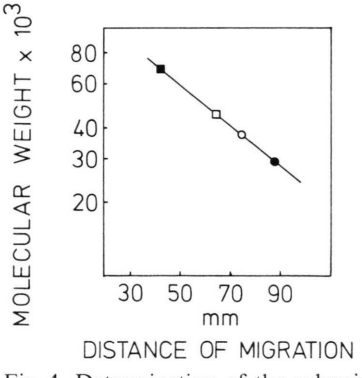

Fig. 4. Determination of the subunit molecular weight of *Aspergillus* aminoacylase by SDS gel electrophoresis. ■ Human serum albumin; □ ovalbumin; ● carboanhydratase; ○ aminoacylase. M_r of 37000 was found for the subunit of acylase.

Kinetic studies

The kinetic parameters of the hydrolysis of some N-chloroacetyl amino acids catalyzed by *Aspergillus* acylase are summarized in Table III. The k_{cat}/K_M quotient gives a relative measure of the substrate specificity of the enzyme. The observed values show, that acyl derivatives of aromatic amino acids and of amino acids with unbranched hydrophobic side chains are prefered substrates. Acetyl derivatives of glutamic and aspartic acid and dichloroacetyl derivatives of glycine and leucine are not accepted by the enzyme as substrates. A slow hydrolysis of acetylalanine, acetylglutamine and acetyllysine was found. A comparison of the relative activity of *Aspergillus* and pig kidney aminoacylase with several substrates is shown in Table IV. Considerable differences of the activity of the two enzymes with the substrates tested are observed. *Aspergillus* acylase has *no peptidase activity* in contrast to the pig kidney enzyme. Another striking difference between the two enzymes is, that only *Aspergillus* acylase can hydrolyse Cl-Ac-Glu-PABA.

The pH dependence of the enzymatic hydrolysis of chloroacetylalanine, chloroacetylphenylalanine and acetylalanine is shown in Fig. 5. The hydrolysis of acetylalanine and chloroacetylalanine has a maximum at pH 8.5 in contrast to the hydrolysis of chloroacetylphenylalanine, which shows a maximum at pH 6.0.

Effects of chelating agents and metal ions on the aminoacylase activity

The activity of *Aspergillus* aminoacylase depends on the presence of metal ions. This conclusion may be drawn from inactivation experiments with metal chelating agents such as *o*-phenanthroline and EDTA. Complete inactivation of the enzyme is observed after dialysis for 24 h against a 0.03 M phos-

Table III. Kinetic parameters of the hydrolysis of N-chloroacetyl amino acids catalyzed by *Aspergillus* aminoacylase.

N-chloro-acetyl	$K_m \times 10^3$ [M]	$V_{max} \times 10^5$ [M × sec^{-1}]	$k_{cat} \times 10^{-2}$ [sec^{-1}]	$k_{cat}/K_m \times 10^{-5}$ [$M^{-1} \times sec^{-1}$]
– Ala	6.3	0.42	1.8	0.28
– Leu	33.0	1.1	1.0	0.03
– Met	1.5	1.1	22.0	15
– Norleu	1.6	1.7	7.1	4.4
– Phe	0.7	8.6	36.0	54
– Trp	1.0	0.086	19.0	19.0

Table IV. Comparison of the relative activity of *Aspergillus* and pig kidney aminoacylase with different substrates.

Substrate	Concentration [mM]	*Aspergillus* aminoacylase	Pig kidney aminoacylase
chloroacetylalanine	7.1	100 [a]	100 [a]
chloroacetylmethionine	7.1	400	480
chloroacetylnorleucine	2.1	207	120
chloroacetylleucine	2.1	26	96
chloroacetylphenylalanine	3.5	325	5
chloroacetyltryptophan	2.1	125	0
acetylglutamic acid	8.2	0	21
acetylaspartic acid	8.2	0	0
acetylglutamine	8.2	13	4
acetylalanine	8.2	14	7
acetyllysine	8.2	3	0
dichloroacetylglycine	4.1	0	1
dichloroacetylleucine	4.1	0	3
dichloroacetylnorleucine	4.1	4	69
dichloroacetylalanine	4.1	0.7	2

[a] $V_{max} = 4.2$ μM × sec^{-1}.

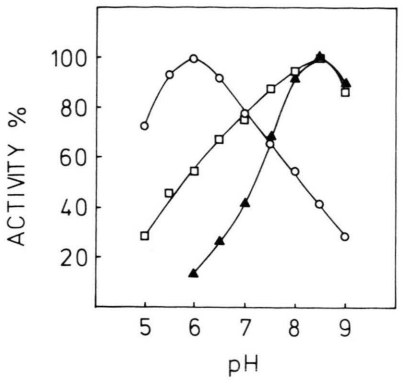

Fig. 5. pH dependence of *Aspergillus* aminoacylase. □ Chloroacetylalanine (5.26 mM); ○ chloroacetylphenylalanine (5.26 mM); ▲ acetylalanine (8.1 mM).

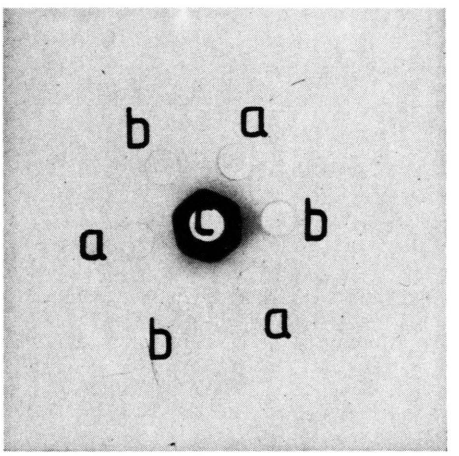

Fig. 6. Ouchterlony diffusion with metal free and metal containing pure aminoacylase. a) Enzyme which was dialyzed against 10 mM EDTA; b) native, metal containing enzyme; c) antiserum (monospecific).

Table V. Effect of metal ions on the activity of aminoacylase (% residual activity).

Metal ion	pH 6		pH 5
	5.6×10^{-4} M	8.7×10^{-4} M	8.7×10^{-4} M
Zn^{2+}	116	112	129
Cd^{2+}	4.5	3.0	36
Co^{2+}	176	165	302
Ni^{2+}	31	25	64
Mn^{2+}	43	19	87
Cu^{2+}	0	0	0
Fe^{2+}	93	–	–
Mg^{2+}	113	101	110

phate buffer containing 10 mM EDTA. Immunochemical experiments have shown that the metal ions are not essential for maintaining the native conformation of the enzyme. Fig. 6 illustrates the result of a typical Ouchterlony immunodiffusion experiment. Inactivation of the enzyme by chelating agents is completely reversible; activity can easily be restored by addition of zinc or cobalt ions without a lag period.

The metal analysis of an enzyme preparation, which was extensively dialyzed against a zinc free buffer, gives a product containing 5.86 ± 0.3 atoms/enzyme mol (M_r calculated from amino acid analysis). This value is in accordance with the presence of three atoms zinc per subunit. However, at the present time we do not assume, that all three zinc atoms are essential for the catalytic activity. Further studies will clarify this question.

The effects of different metal ions on the active enzyme are demonstrated in Table V. The results of these experiments support the suggestion, that aminoacylase from *Aspergillus oryzae* is a metallo enzyme. Aminoacylase can be significantly activated by Co^{2+} ions. This conclusion can be drawn from activation experiments with chloroacetyl alanine as substrate (Table V). Activation of Zn^{2+} dependent amide hydrolases by Co^{2+} ions is a common phenomenon of several peptidases, which is not yet understood at the present time. Experiments to prepare a pure Co^{2+} acylase by a Zn^{2+}/Co^{2+} exchange and the investigation of the properties and catalytic activity of this preparation are under way.

Comparison of pig kidney and Aspergillus oryzae aminoacylase

A comparison of some kinetic and chemical properties of the mammalian pig kidney acylase and the microbial enzyme from *Aspergillus oryzae* is given in Table VI. Both enzymes are metallo proteins containing essential zinc ions; both enzymes may be activated by Co^{2+} ions as it is observed with many zinc dependent peptidases. We have good reasons to asume that an exchange of zinc and cobalt takes place, the cobalt enzyme being essentially more active than the zinc enzyme. Treatment of both enzymes with 10^{-2} M diethylpyrocarbonate results in an inactivation of the pig kidney acylase and of the *Aspergillus* enzyme. In contrast to the pig kidney acylase the *Aspergillus* enzyme does neither react

Table VI. Comparison of the kinetic and chemical properties of pig kidney and *Aspergillus oryzae* aminoacylase.

	Aspergillus oryzae aminoacylase	Pig kidney aminoacylase	References
Molecular weight	73 200	85 500	[14]
Subunits	2	2	[14]
Metal ions (Zn^{2+})	6	2	[15]
SH-groups	0	4	[14]
Inhibition by N-α-*p*-tosyl-L-lysine chloromethyl ketone HCl	−	+	[11]
Inhibition by diethylpyrocarbonate	+	+	[16]
Inactivation by metal chelating agents	completely reversible	completely reversible	[15]
pH-optimum (chloroacetylalanine)	8.5	8.0	[30]
1/2 Cystein residues	4	12	[14]
Tryptophan residues	6	12	[14]
$K_m \times 10^3$ mol/l (chloroacetylalanine)	6.3	6.6	[30]
Spec. activity U/mg (chloroacetylalanine)	319 (pH 8.0)	250 (pH 7.8)	[14]
Peptidase activity	−	+	[30]
Cl-Ac-Glu-PABA hydrolysis	+	−	
Heat stability	60 °: denaturation	60 °: denaturation	[11]
Activation by Co^{2+}	+	+	[11]

with thiol reagents (iodoacetamide and N-ethylmaleimide) nor with N-α-*p*-tosyl-L-lysine chloromethyl ketone. Only the mammalian enzyme contains essential SH-groups. Phenylmethylsulfonyl fluoride, a specific irreversible inhibitor of proteinases does not inhibit the activity of the microbial enzyme.

The inhibition experiments raise the question to the essential amino acids at the active site of *Aspergillus* aminoacylase, which cannot be answered conclusively at the present time. This problem is the topic of further investigations.

Acknowledgements

We thank Mrs. Ziegler, Karlsruhe for the zinc analysis. We are indepted to Mr. Th. Stein and Mr. R. Böcher for synthesizing the substrates.

[1] S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem. **194,** 455–470 (1952).
[2] H. T. Hanson and R. Kleine, Hoppe Seyler's Z. Physiol. Chem. **315,** 208–214 (1959).
[3] Chem. Abstr. **Vol. 65,** 11 312 (1966).
[4] Chem. Abstr. **Vol. 82,** 94 841 t (1975).
[5] Amano Enzymes, Technical Bulletin 70, Amano Pharmaceutical Co. Ltd. Nagoya, Japan.
[6] J. P. Greenstein and M. Winitz, Chemistry of the Amino Acids, pp. 1753–1767, J. Wiley & Sons Inc. 1961.
[7] J. L. Brown and W. K. Roberts, J. Biol. Chem. **251,** 1009–1014 (1966).
[8] S. Tsunazawa, K. Narita, and K. Ogata, Proc. Japan Acad. **46,** 960–967 (1970).
[9] S. Tsunazawa, K. Narita, and K. Ogata, J. Biochem. (Tokyo) **77,** 89–98 (1975).
[10] W. Gach and J. L. Brown, J. Biol. Chem. **253,** 5012–5018 (1978).
[11] W. Kördel, Thesis, University of Marburg 1976.
[12] H. F. Bruns and Chr. Schulze, Biochem. Z. **336,** 162–181 (1962).
[13] W. Kördel and Fr. Schneider, Hoppe Seyler's Z. Physiol. Chem. **357,** 1109–1115 (1976).
[14] W. Kördel and Fr. Schneider, Biochim. Biophys. Acta **445,** 446–457 (1976).
[15] W. Kördel and Fr. Schneider, Z. Naturforsch. **32 c,** 342–344 (1977).
[16] W. Kördel and Fr. Schneider, Z. Naturforsch. **32 c,** 337–341 (1977).
[17] E. Ronwin, J. Org. Chem. **18,** 1546–1553 (1953).
[18] H. Krebs and W. Schumacher, Chem. Ber. **99,** 1341–1346 (1966).
[19] Y. Kameda, Y. Kimura, and H. Yamazoe, Kanazawadaigaku Yakugakubu Kenkyu Nempo 6, 16 (1955); zitiert nach Y. Kameda, T. Nakabayashi, S. Kanatomo, and S. Kitade, Chem. Pharm. Bull. **26,** 3785–3791 (1978).
[20] A. Szewczuk and M. Wellman-Bednawska, Clin. Chim. Acta **84,** 27–31 (1978).
[21] H. R. Maurer, Disc Electrophoresis, p. 44–45, de Gruyter Verlag 1971.
[22] G. Blobel and B. Dobberstein, J. Cell Biol. **67,** 835–851 (1975).
[23] U. K. Laemmli, Nature **227,** 680–685 (1970).
[24] K. Lorentz, Biochemistry **76,** 214–220 (1976).
[25] H. Matsubara and R. M. Sasaki, Biochem. Biophys. Res. Commun. **35,** 175–178 (1969).
[26] T. Dévény and J. Gergely, Amino Acids, Peptides and Proteins, p. 162, Elsevier Scientific Publishing Company Amsterdam, London, New York 1974.
[27] G. L. Ellman, Arch. Biochem. Biophys. **82,** 70–77 (1959).
[28] A. H. Reisner, P. Nemes, and C. Bucholtz, Anal. Biochem. **64,** 509–516 (1975).
[29] J. C. Bailey, Techniques in Protein Chemistry, p. 341, Elsevier, Amsterdam, London, New York 1967.
[30] W. Kördel and Fr. Schneider, Hoppe Seyler's Z. Physiol. Chem. **356,** 915–920 (1975).
[31] K. R. Rao, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem. **198,** 507–523 (1952).
[32] O. Ouchterlony, Arkiv Kemi **1,** 43–54 (1949).