LITERATURE CITED

1. H. Seto, K. Furihata, and H. Yonehara, J. Antibiot., 29, 595 (1976).
2. K. A. Watanabe, D. H. Hollenberg, and J. J. Fox, J. Antibiot., 29, 597 (1976).
3. P. C. Wyss, J. Kiss, and W. Arnold, Helv. Chim. Acta, 58, No. 6, 1847 (1976).

ISOLATION AND PURIFICATION OF AN AMINOACYLASE FROM *Aspergillus oryzae*

T. A. Solov'eva and V. M. Stepanov

UDC 511.152.351

An aminoacylase (EC 3.5.1.14) has been isolated from a surface culture of the fungus *Aspergillus oryzae* (amilorizin P10X) with a 764-fold degree of purification, an activity yield of 32.7%, and a specific activity in relation to the hydrolysis of N-acetyl-D,L-methionine of 99.3 a.u./o.u. The scheme of the purification of the aminoacylase from *Aspergillus oryzae* includes: extraction at pH 6.7, precipitation with ammonium sulfate (30 and 80% saturation), gel filtration on Acrilex P-150 (pH 7.5), ion-exchange chromatography on amino-Silochrom Cx-1,5 (mean pore radius 790 Å); the sorption of the enzyme takes place at pH 6.2 and elution with 0.05 M borate buffer, pH 8.0; ion-exchange chromatography on AH-Sepharose 4B at pH 8.0, with elution by a stepwise increase in the concentration of sodium chloride to 0.25 M; and, finally, gel filtration on Sephadex G-200 (pH 8.0). According to the results of disk electrophoresis in 7.5% polyacrylamide gel in a Tris-glycine systems of buffers with a separation pH of 8.9 in the presence of Co^{2+} ions (10^{-5} M) of the *Aspergillus oryzae* aminoacylase, two components possessing enzymatic activity were detected, with R_f 0.53 (major component) and R_f 0.63 (minor component).

The use of an enzyme hydrolyzing acyl-L-(amino acids) — aminoacylase (EC 3.5.1.14) — for the separation of amino acid racemates obtained synthetically into their optical antipodes is well known. Until recently, the asymmetric hydrolysis of various N-acetyl derivatives of amino acids was carried out with the aid of aminoacylase of animal origin [1].

A partially purified aminoacylase of microbial origin was first obtained from the mycelium from a mold fungus, strain No. 9 of *Aspergillus oryzae* [2]. The possibility was shown of using the *Aspergillus oryzae* aminoacylase for the continuous separation of N-acetyl-D,L-(amino acid)s [3, 4]. Recently, it has been possible to obtain an aminoacylase from a commercial preparation of *Aspergillus oryzae* which is homogeneous according to electrophoresis in polyacrylamide gel [5]. The purification of the enzyme included the thermal treatment of the intiial preparation, subsequent precipitation with 5% polyethyleneimine and ammonium sulfate, gel filtration on Sephadex G-150, and preparative disk electrophoresis. A 201-fold purification was achieved with a yield of 14%.

We have previously obtained an aminoacylase from a surface culture of *Aspergillus oryzae* (amilorizin P10X) by the extraction of the initial preparation at pH 8.0, precipitation with ammonium sulfate (80% saturation), ion-exchange chromatography on DEAE- and ECTEOLA-celluloses, gel filtration on Sephadex G-200, and affinity chromatography. The specific activity of the preparation rose by a factor of 800, amounting to 36.5 μmole of L-methionine/mg/min (N-acetyl-D,L-methionine was used as the substrate. We have studied some physicochemical properties of the enzyme preparation obtained: action pH optimum, dependence of the stability on the pH, dependence of the activity of the enzyme on the concentration of Co^{2+} ions, substrate specificity [6].

In the production of a highly purified preparation of the aminoacylase, we came up against a number of difficulties connected with the fact that a surface culture of *Aspergillus oryzae* contains, in addition to a multiplicity of pigments and a large number of products of the

All-Union Scientific-Research Institute for the Genetics and Breeding of Industrial Microorganisms, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 619-624, September-October, 1983. Original article submitted August 16, 1982.

 0009-3130/83/1905-0582$07.50

TABLE 1. Isolation of the Aminoacylase from a Surface Culture of the Fungus *Aspergillus oryzae*

Stage of purification	Protein, o.u. (E_{280})	Specific activity, a.u./o.u.	Total activity, a.u.	Degree of purification, times	Activity yield, %
Extraction of the initial preparation in 0.2 M buffer	55100	0,13	7160	1	100
Precipitation with ammonium sulfate (30 and 80% saturation)	—	—	—	—	—
Gel filtration on Acrilex P-150	4945	1,24	6130	9 5	85,6
Chromatography on amino-Silochrom Cx-1,5	630	8,2	5170	63	72
Chromatography on AH-Sepharose 4B	83 5	32,30	2700	248	37,7
	23,6	99,30	2340	764	32,7

vital activity of the organisms, enzymes hydrolyzing the matrices of ion-exchange materials based on cellulose — Sephadex and Sepharose — cellulases and dextranases. This circumstance formed the basis for the development of a more convenient method of isolating the aminoacylase of microbial origin, and it is to this to which the present communication is devoted.

The scheme of the isolation of the aminoacylase from a surface culture of *Aspergillus oryzae* includes extraction, precipitation with ammonium sulfate at 30% and 80% saturation, gel gel filtration on Acrilex P-150, ion-exchange chromatography on amino-Silochrom and AH-Sepharose 4B and gel filtration on Sephadex G-200 (Table 1).

An extract of the initial preparation was precipitated with ammonium sulfate (30% saturation), which enabled it to be freed from a number of impurities, and then the enzyme was isolated from the supernatant liquid by precipitation with ammonium sulfate at 80% saturation. The fractionation by ammonium sulfate prevented the development of microorganisms which considerably complicates the subsequent purification of the enzyme. Pigments were separated by gel filtration on Acrilex P-150, the polyacrylamide matrix of which is resistant to the action of cellulases and dextranases. The resulting solution of aminoacylase was chromatographed on amino-Silochrom Cx-1,5 — an anion-exchange material based on macroporous silica the matrix of which is also resistant to the action of carbohydrases and microorganisms. The optimum pH for the binding of the acylase to the amino-Silochrom was 6.2. Under these conditions, more than 80% of the impurity proteins were not retained by the anion-exchanger and issued from the column when it was washed with a buffer solution at pH 6.2 (Fig. 1). The enzyme was eluted with 0.05 M borate buffer, pH 8.0. The two subsequent stages of the purification of the aminoacylase — gel filtration on Acrilex P-150 and chromatography on amino-Silochrom Cx-1.5 — enabled not only the bulk of the pigments and impurity proteins but also the carbohydrases to be eliminated, which permitted the subsequent use of the usual methods of protein chemistry. In the following stage, the purification of the aminoacylase was carried out by chromatography on AH-Sepharose 4B. The adsorption of the aminoacylase was carried out in 0.05 M borate buffer, pH 8.0 (Fig. 2). An additional elimination of impurity proteins was performed by stepwise elution with a gradual increase in the ionic strength of the eluent. The washing of the column of AH-Sepharose 4B with a 0.12 M solution of NaCl, pH 8.0, permitted the elimination of small amounts of pigment still retained in the preparation. The aminoacylase began to be desorbed by a 0.19 M solution of NaCl, but the bulk of the enzyme was eluted by 0.25 M NaCl in 0.05 M borate buffer, pH 8.0 (Fig. 2). The concluding stage of the purification of the *Aspergillus oryzae* aminoacylase (with simultaneous desalting) was gel filtration on Sephadex G-200 (Fig. 3).

The proposed scheme for the isolation of an aminoacylase from a surface culture of *Aspergillus oryzae* permitted a 764-fold purification of the enzyme with an activity yield of 32% and the achievement of a specific activity of 99.3 a.u./o.u. (Table 1). The disk electrophoresis in 7.5% polyacrylamide gel, pH 8.9, of the preparation obtained showed two protein bands possessing activity in the hydrolysis of N-acetyl-D,L-methionine: a major component with R_f 0.53, and a minor component with R_f 0.63 (Fig. 4).

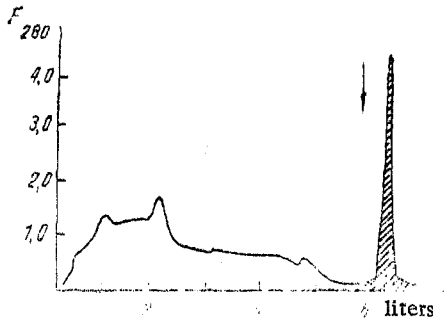

Fig. 1

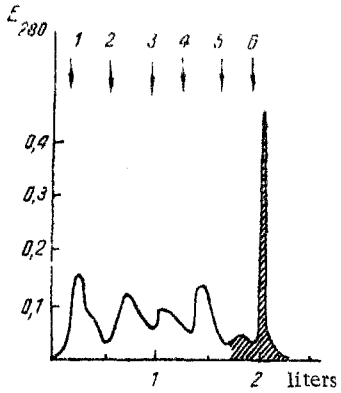

Fig. 2

Fig. 1. Chromatography of the *Aspergillus oryzae* aminoacylase on amino-Silochrom Cx-1,5. Column 6 × 8 cm, 4945 o.u. of aminoacylase with a specific activity of 1.24 a.u/o.u., 0.1 M potassium phosphate-borate buffer, pH 6.2. The arrow shows the beginning of the elution of the enzyme by 0.05 M borate buffer, pH 8.0. The hatched peak contains the active protein.

Fig. 2. Chromatography of the *Aspergillus oryzae* aminoacylase on AH-Sepharose 4B. Column 2.5 × 10 cm, 470 ml of aminoacylase, 630 o.u., with a specific activity of 8.2 a.u./o.u., 0.05 M borate buffer, pH 8.0. The arrows denote a stepwise increase in the concentration of sodium chloride: 1) 0.12 M; 2) 0.14 M; 3) 0.15 M; 4) 0.18 M; 5) 0.19 M; 6) 0.25 M. The hatched peak contains the active protein.

EXPERIMENTAL

In the experiments, a surface culture of the fungus *Aspergillus oryzae* — amilorizin P10X — was used as the initial enzyme preparation. We also used Silochrom of type 1.5 (0.8-0.15 fraction) with a mean pore radius of 790 Å, γ-aminopropyltriethoxysilane from Reakhim (USSR), N-acetyl-D,L-methionine (GFR), Acrilex P-150 from Reanal (Hungary), AH-Sepharose 4B and Sephadex G-200 (Sweden), and a PMR-30 ultramembrane (Holland).

Preparation of Amino-Silochrom. Silochrom Cx-1,5 was added to 750 ml of a 5% solution of γ-aminopropyltriethoxysilane in toluene, and the mixture was carefully stirred. Then it was heated in a glycerol bath (110°C) under reflux for 12 h with stirring every 30 min. The excess of γ-aminopropyltriethoxysilane was eliminated by washing the amino-Silochrom successively with toluene (2 liters), acetone (2 liters), distilled water (2 liters), 1 M HCl (1 liter), and distilled water until chloride ions had been eliminated (check with $AgNO_3$). The preparation was dried at 70°C. The capacity of the dry preparation of amino-Silochrom obtained was 0.95 meq/g [7].

The aminoacylase activity was determined from the hydrolysis of N-acetyl-D,L-methionine as standard substrate (I). A mixture containing 0.2 ml of a 0.1 M solution of N-acetyl-D,L-methionine, pH 8.0, 0.2 ml of a $5 \cdot 10^{-4}$ M solution of Co^{2+}, pH 8.0, 0.5 ml of a 0.05 M borate buffer, pH 8.0, and 0.1 ml of a solution of the enzyme was kept at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of a 2% solution of ninhydrin in methylcellosolve and $2 \cdot 10^{-3}$ M stannous chloride in 1 M acetate buffer, pH 5.0, and the mixture was carefully stirred, kept at 100°C for 15 min, treated with 5 ml of 60% ethanol, and stirred again, and its optical density was measured at 750 nm. In a control sample, the enzyme was added after the ninhydrin solution had been added to the reaction mixture. The ninhydrin solution was considered suitable for use when the color of a mixture of 1 ml of the ninhydrin solution and 1 ml of a standard solution of L-norleucine (with a concentration of 0.5 μM of the amino acid in 1 ml of 0.05 M borate buffer, pH 8.0), after being kept at 100°C for 15 min and after the addition of 5 ml of 60% ethanol to the mixture gave an absorption of 1.0 at 750 nm. As the activity unit (a.u.) we took the amount of enzyme which, under standard conditions, gave an increase of 1.0 at 750 nm in 1 min. The specific activity was calculated as a.u/ml:

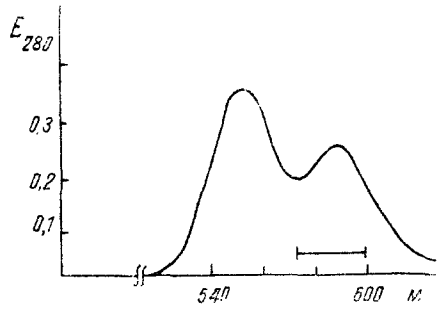

Fig. 3. Gel filtration of the *Aspergillus oryzae* aminoacylase on Sephadex G-200. Column 5 × 100 cm, 28 ml of enzyme, 27 o.u. with a specific activity of 43.3 a.u./o.u., 0.5 M borate buffer, pH 8.0. The fraction indicated contains the active protein.

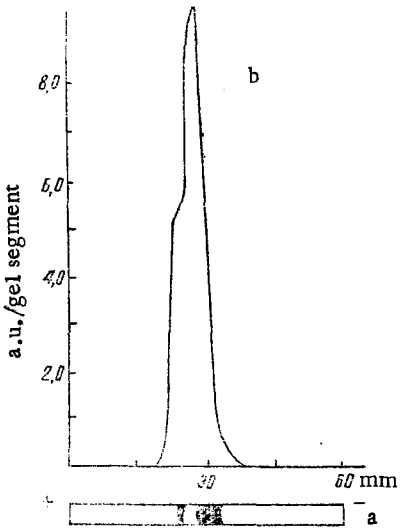

Fig. 4. Disk electrophoresis of purified *Aspergillus oryzae* aminoacylase in polyacrylamide gel: a) staining of the gel with Coomassie Blue (20 μg of the enzyme was used in the experiment); b) determination of activity with N-acetyl-D,L-methionine as substrate.

E_{280} = a.u./o.u., where a.u./ml is the number of activity units in 1 ml of the enzyme solution, and E_{280} is the optical density at 280 nm of the solution of the enzyme added to the reaction mixture, a.u./o.u. being activity units/optical units.

Isolation of the Aminoacylase from a Surface Culture of the Fungus *Asp. oryzae*. A suspension of 110 g of amilorizin P10X in 500 ml of 0.2 M borax (pH of the mixture 6.7) was stirred at 4°C for 19 h. The solid matter was separated off by centrifugation and discarded. The extract obtained, with a specific activity of 0.13 a.u./o.u. was treated with ammonium sulfate to 30% saturation, the pH being maintained at about 7.0 by the addition, with stirring, of a 2 M solution of NaOH. After 20 h at 4°C, the precipitate was separated off by centrifugation and was dissolved in 240 ml of 0.05 M borate buffer, pH 7.5. With the same buffer, 60 ml of this solution was made up to 100 ml and was deposited at the rate of 65 ml/h on a column (5 × 100 cm) of Acrilex P-150 which was equilibrated and washed with the same buffer. Fractions containing enzymatic activity were combined, diluted with an equal volume of 0.1 M potassium phosphate–borate buffer, pH 6.1, to give a final pH of 6.2-6.3, and deposited on a column (6 × 8 cm) of amino Silochrom Cx-1.5 equilibrated with 0.1 M potassium phosphate–borate buffer, pH 6.2. The column was washed with the same buffer until the absorption in the eluate was 0.1. The aminoacylase was eluted with 0.05 M borate buffer, pH 8.0. The column containing the amino-Silochrom was regenerated with a 1 M solution of HCl and was then washed with water to a weakly acidic reaction. Where experiments were performed on a new batch of amino-Silochrom Cx-1.5, for the maximum desorption of the enzyme, in addition to raising the pH, it was necessary to increase the ionic strength of the eluting solution: for the first experiment, 1 M NaCl with pH 8.0, and for the second 0.15 M NaCl with pH 8.0; the desorption of the active protein in the subsequent experiments, as in the case under consideration, was achieved

with a 0.05 M borate buffer, pH 8.0. The active fraction obtained by chromatography on the amino-Silochrome – 470 ml – was deposited on a column (2.5 × 10 cm) of AH-Sepharose 4B equilibrated with 0.05 M borate buffer, pH 8.0. Solutions containing 0.12, 0.14, 0.15, and 0.18 M NaCl in the same buffer were passed through the column successively to elute the impurity proteins. The enzyme was eluted with 0.19 and 0.25 M solutions of NaCl in 0.05 M borate buffer, pH 8.0. This gave 83.5 o.u. of an enzyme with a mean specific activity of 32.3 a.u. a.u./o.u. The column containing the AH-Sepharose 4B was regenerated with a 1 M solution of NaCl in 0.05 M borate buffer, pH 8.0. The active fraction obtained by chromatography on AH-Sepharose 4B was concentrated on a PM-30 ultramembrane at 1.5 atm to 50 ml. The concentrate of aminoacylase (in approximately 25-ml portions) was deposited on a column (5 × 100 cm) of Sephadex G-200 (superfine) equilibrated with 0.05 M borate buffer, pH 8.0, at the rate of 15 ml/h. The enzyme was eluted by the same buffer. This gave 23 o.u. of an aminoacylase with a mean specific activity of 99.6 a.u./o.u. The solution of enzyme obtained was stored at 4°C. All the operations were performed at this temperature.

Disk electrophoresis in 7.5% polyacrylamide gel was carried out in the Tris-glycine system of buffers with a separation pH of 8.9 [8] in the presence of Co^{2+} ions at a final concentration of 10^{-5} M for 1.5 h with a current strength of 3 mA per tube. The experiment was termined when the marker – Bromophenol Blue – was approximately 3 mm from the lower end of the gel. The R_f value of the protein was measured relative to the marker. To determine the activity of the aminoacylase, the column of gel (after the end of electrophoresis) was extracted from the tube, washed twice with 0.05 M borate buffer, pH 8.0 (to eliminate glycine), and cut lengthways into two halves, one of which was stained with a 0.5% solution of Coomasie Blue in 3.5% perchloric acid [9] for 15 min, the excess of dye being eliminated with 7.5% acetic acid. The other hald of the gel was cut into segments each 1.5 mm long. The segments of the gel were placed in numbered test-tubes and were washed three times with 0.05 M borate buffer, pH 8.0. The inner walls of the test-tubes were dried with strips of filter paper, and then 0.6 ml of 0.05 M borate buffer, pH 8.0, 0.2 ml of $5 \cdot 10^{-4}$ M solution of Co^{2+}, and 0.2 ml of 0.1 M N-acetyl-D,L-methionine were added to each tube and they were kept at 37°C for 2 h, after which the gel segments were extracted, and the reaction with ninhydrin was performed in the test-tubes as described above.

SUMMARY

A scheme for the isolation of an aminoacylase from a surface culture of the mold fungus *Aspergillus oryzae* has been worked out. Gel filtration on Acrilex P-150, ion-exchange chromatography on amino-Silochrome Cx-1.5 and on AH-Sepharose 4B, and gel filtration on Sephadex G-200 have given the aminoacylase with an activity yield of 32.7%, a degree of purification of 764-fold, and a specific activity of 99.3 a.u./o.u. Disk electrophoresis in polyacrylamide gel has shown the presence of two components of the enzyme possessing enzymatic activity, with R_f 0.53 (major component) and R_f 0.63 (minor component).

LITERATURE CITED

1. J. P. Greenstein and M. Winitz, Chemistry of the Amino Acids, Wiley, New York (1961).
2. T. Tosa, T. Mori, N. Fuse, and I. Chinata, Enzymologia, 31, No. 4, 214 (1966).
3. I. Chibata, in: Proceedings of the International Symposium on the Conversion and Manufacture of Food Stuffs by Microorganisms, Tokyo, Japan (1971), p. 75.
4. I. Chibata, T. Tosa, T. Sato, T. Mori, and Y. Matsuo, in: Proceedings of the 4th International Fermentation Symposium: Fermentation Technology Today, Tokyo (1972), p. 383.
5. I. Gentzen, H.-G. Löfler, and F. Schneider, Z. Naturforsch., C35, Nos. 7-8, 544 (1980).
6. L. S. Lobareva, T. A. Solov'eva, Ya. I. Lapuk, L. A. Lyublinskaya, and V. M. Stepanov, Methods of Obtaining Highly Purified Enzymes. Abstracts of an All-Union Symposium [in Russian], Vilnius (1978), p. 83.
7. L. M. Ginodman, in: Modern Methods in Bicochemistry [in Russian], Moscow (1964), p. 46.
8. B. J. Davis, Ann. N.Y. Acad. Sci., 121, 404 (1964).
9. I. B. Holbrook and A. G. Leaver, Anal. Biochem., 75, 634 (1976).