tion of mitochondria with phospholipase D in the presence of methanol, glycerol, or ethanolamine changes the phospholipid composition of the mitochondria and leads to the formation of phosphatidylmethanol, phosphatidylglycerol, and high concentrations of lysophosphatidylethanolamine. The incubation of mitochondria with phospholipase D causes mainly a considerable decrease in the activities of cytochrome c oxidase and NADH-cytochrome c reductase.

LITERATURE CITED

1. A. Casu and B. Fleisher, Fed. Proc., 23, 486 (1964).
2. J. Machinist and T. R. Singer, J. Biol. Chem., 240, 3182 (1965).
3. B. Fleisher, A. Casu, and S. Fleisher, Biochem. Biophys. Res. Commun., 24, 189 (1966).
4. S. Fleisher, A. Casu, and B. Fleisher, It. J. Biochem., 26, 278 (1977).
5. J. C. Wilschut and G. L. Scherphof, Biochem. Biophys. Acta, 256, 91 (1974).
6. A. Sasu, A. Saito, B. Fleisher, and S. Fleisher, It. J. Biochem., 26, 298 (1977).
7. A. S. Denes and N. Z. Stansev, Canad. J. Biochem., 56, 905 (1978).
8. M. Waite and P. Sisson, Biochemistry, 10, 2377 (1971).
9. G. P. Brierly and A. J. Merola, Biochem. Biophys. Acta, 64, 205 (1962).
10. G. Gercken and W. Witt, Acta Biol. Med. Ger., 36, 837 (1977).
11. D. Johnson and H. Lardy, Methods in Enzymology, Vol. 10, R. W. Estabrook and M. Pullman, eds., Academic Press, New York (1967), p. 94.
12. E. M. Davidson and C. Long, Biochem. J., 69, 458 (1958).
13. D. C. Wharton and A. Tzagoloff, Methods in Enzymology, Vol. 10, R. W. Estabrook and M. Pullman, eds., Academic Press, New York (1967), p. 245.
14. S. Fleisher and B. Fleisher, Methods in Enzymology, Vol. 10, R. W. Estabrook and M. Pullman, eds., Academic Press, New York (1967), p. 406.
15. V. I. Svetachev and V. E. Vaskovsky, J. Chromatogr., 67, 376 (1972).
16. O. H. Lowry, N. J. Rosenbrough, A. I. Farr, and R. J. Randall, J. Biol. Chem., 193, 265 (1951).

ISOLATION AND CHARACTERIZATION OF AN L-AMINO ACID ACYLASE FROM *Aspergillus oryzae*

M. K. Malinka, V. M. Stepanov, and L. S. Lobareva

UDC 577.154.52

A scheme of isolating a highly purified L-amino acylase from *Aspergillus oryzae* is described which excludes extraction of the enzyme from the preparation "Amilorizin," fractionation with ethanol, chromatography on DEAE-cellulose, and gel filtration through Sephadex G-200 and Bio-Gel P-300. The enzyme, as purified 1240-fold, has a molecular weight of 118,000, apparently consists of two subunits with a molecular weight of 60,000, is stable in the pH range of 7-10 and has an optimum pH of 8.9 and a pI of 4.0. Its amino acid composition has been determined and its substrate specificity has been studied. The acylase is a metalloenzyme: Co^{2+} ions in concentrations of 10^{-4}-$5 \cdot 10^{-5}$ M increase the rate of hydrolysis of N-acetyl-L-amino acids three- to fourfold. It shows differences in its molecular and functional properties from acylase I obtained from porcine kidney.

L-Amino acid acylase (E.C. 3.5.1.14) hydrolyses the amide bonds of N-acetyl-L-amino acids. This enzyme has been detected in various microorganisms — fungi, bacteria, and yeasts [1, 2]. The acylases of microscopic fungi are used for the isolation of L-amino acids from the racemates obtained by chemical synthesis. Unpurified or partially purified preparations of *Aspergillus oryzae* are used for this purpose [3-5]. However, hitherto there has been no information on the properties of pure acylases of microscopic fungi. The purification of fungal acylases is made difficult by the fact that the initial preparations contain a multiplicity of other proteins, enzymes, and pigments. For example, a surface culture of

All-Union Scientific-Research Institute of the Genetics and Breeding of Industrial Microorganisms, Moscow.

Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 370-379, May-June, 1982. Original article submitted July 28, 1981.

0009-3130/82/1803-0341$07.50

TABLE 1. Isolation of the Acylase *Aspergillus oryzae*

Stage of purification	Protein, A_{280}	Activity		Activity yield, %	Degree of purification
		specific, μmole/h·A_{280}	total, μmole/h		
1. Initial preparation	90 g	2,5	225 000	100	1
Extraction with 0.1 M borate buffer, pH 7.0	40 g	3,1	124 000	55	1,24
2. Fractionation with ethanol	3100	30	93 000	41	12.0
3. Chromatography on DEAE-cellulose, pH 8.0	182	143	26 000	11,6	57,2
4. Gel filtration on Sephadex G-200	25	740	18 500	8,2	296
5. Chromatography on DEAE-cellulose, pH 5.7	4	2 300	9 200	4,1	920
6. Gel filtration on Bio-Gel P-300	2,9	3 100	9 000	4,0	1240

After stages 4, 5, and 6 the enzyme solutions were concentrated by ultrafiltration in an Amicon apparatus with a UM-10 membrane.

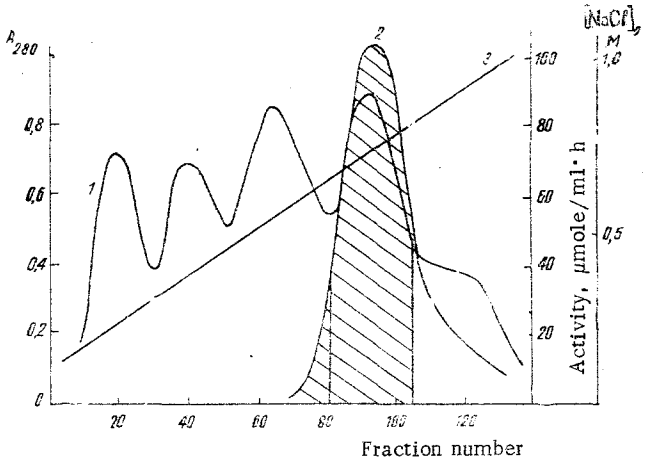

Fig. 1. Chromatography of an unpurified acylase preparation on a column (2.5 × 40 cm) of DEAE-cellulose at pH 8.0: 1) A_{280}; 2) activity in 1 ml of solution; 3) concentration of NaCl.

Aspergillus oryzae produces an acid proteinase, a cellulase, and a peptidase and is rich in amylolytic enzymes.

The aim of the present investigation was to obtain and study the properties of the L-amino acid acylase from *Aspergillus oryzae*.

A scheme for the isolation of the amylase has been developed which includes six stages (Table 1). After the extraction of the enzyme from "Amilorizin" — a surface culture of *Aspergillus oryzae* — with 0.1 M borate buffer, pH 7.0, fractional precipitation was carried out with ethanol in the range of concentrations of from 30 to 50%. This operation gives a tenfold purification with an 80% yield and permits the elimination of the bulk of the pigments. Ethanol fractionation is considerably more effective than salting out with ammonium sulfate. Substantial purification of the enzyme was achieved by chromatography on DEAE-cellulose at pH 8.0. This process led to a 4.2-fold rise in activity, with a yield of 30%. Then gel filtration was carried out on a column of Sephadex G-200 and chromatography on DEAE-cellulose at pH 5.7. After gel filtration the degree of purification of the acylase had increased 5.3-fold, the yield amounting to 70%. By chromatography on DEAE-cellulose at pH 5.7 it was possible to separate acidic protein impurities. In spite of the fact that the acylase is unstable even in weakly acid solutions, chromatography on DEAE-cellulose at pH 5.7 is

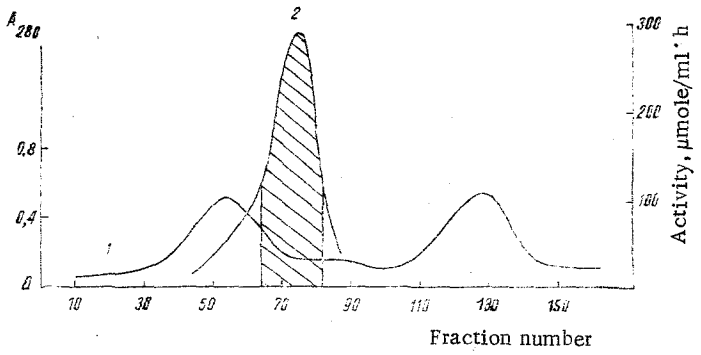

Fig. 2. Gel filtration of an acylase preparation on a column (5 × 100 cm) of Sephadex G-200: 1) A_{280}; 2) activity of 1 ml of the fraction.

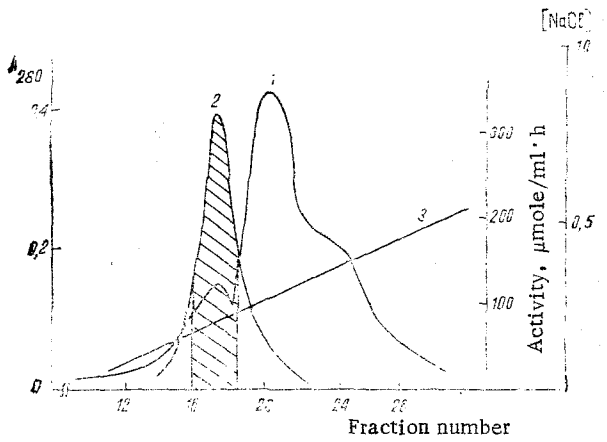

Fig. 3. Chromatography of an acylase preparation on a column of DEAE-cellulose at pH 5.7. Symbols the same as in Fig. 1.

very effective (see Fig. 3). When the enzyme was eluted with an NaCl gradient, 85% of the inactive protein was removed. In the preparations obtained, electrophoresis in PAAG showed, in addition to the main strong band, three minor bands electrophoretically more mobile. Final purification was achieved by gel filtration on Bio-Gel P-300. The hatched part of the peak in Fig. 4 contained 97% of the activity and 72% of the total amount of protein deposited on the column.

As a result of the multistage process described above, the enzyme was purified by a factor of 1240, with a yield of 4%. The relatively low yield is due to the large losses in chromatography on DEAE-cellulose, and also to the inactivation of the diluted enzyme solutions on prolonged concentrations in the ultrafiltration apparatus.

The homogeneity of the acylase was shown by disk electrophoresis in 7.5% PAAG at a separation pH of 9.0 (Fig. 5). Apart from the main active component, there was a weak active electrophoretically more mobile protein band. On isoelectric focusing at pH 3-9.5 and 2-5, a single protein zone with pI 4.0, obviously corresponding to the acylase, was detected.

The molecular weight of the enzyme, determined by gel filtration on Sephadex G-200, is 118,000. Table 2 gives the amino acid composition of the acylase. In a determination of the molecular weight of the polypeptide chain by electrophoresis with sodium dodecyl sulfate (SDS) in 5% PAAG a band of predominating intensity with a molecular weight of 110,000 ± 5000 and a weak band with a molecular weight of 60,000 were detected. The low-molecular-weight component may be either a subunit or a product of the limited proteolysis of the acylase by protease impurities. In this respect, the stage of preparing the enzyme sample for electrophoresis by heating it with SDS could present a risk. It leads to the denaturation of the enzyme, which becomes more accessible for proteolysis by protease impurities. The dissolu-

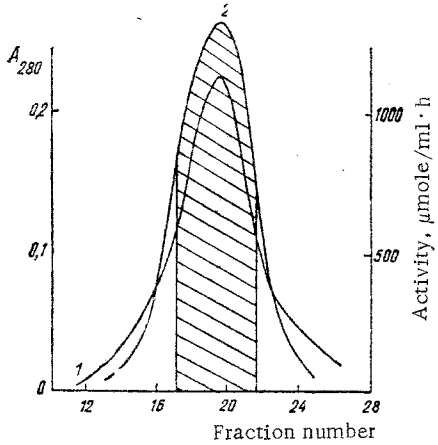

Fig. 4

Fig. 5

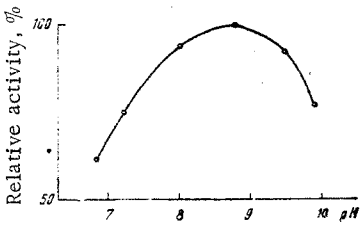

Fig. 6

Fig. 4. Gel filtration of the acylase on a column (1.4 × 100 cm) of Bio-Gel P-300. Symbols the same as in Fig. 2.

Fig. 5. Disk electrophoresis of the acylase from *Asp. oryzae* at pH 9.0.

Fig. 6. pH dependence of the activity of the acylase.

tion of a sample of the acylase in aqueous phenol with subsequent precipitation by acetone ensures the complete denaturation of possible protease impurities. On electrophoresis with SDS of the acylase treated in this way, two protein bands with the same distribution of intensities of coloration as without the phenol treatment were detected.

Limited proteolysis could also take place in the course of purification of the acylase, particularly through the action of serine proteases. In view of this, a special experiment was performed to isolate the enzyme during which all the stages of purification were carried out in the presence of a special serine protease inhibitor — phenylmethanesulfonyl fluoride.

However, as electrophoresis in the presence of SDS showed, in spite of the blockage of the serine proteases two protein bands were observed in the preparation, as before. It must be mentioned that the possibility is not excluded of proteolysis in the growth and treatment of a surface culture of *Aspergillus oryzae*.

We have compared the amino acid compositions of the substances present in the bands that are observed on electrophoresis in the presence of SDS. After electrophoresis, the protein bands were cut out from the gel, and then the proteins were eluted by three different methods: by electrodialysis, with water, and with 0.15 M NaCl containing 0.1% of SDS [16]. The amino acid compositions of the proteins of both bands calculated to 100 residues were identical.

In view of this, we assume that the two bands observed on electrophoresis with SDS most probably represent a dimer and a monomer with molecular weights of 120,000 and 60,000, respectively.

Boiling protein samples with 1% SDS, 8 M urea, and 0.1% mercaptoethanol for 2 minutes as recommended by Weber and Osborn [15] is probably insufficient for the complete dissociation of some proteins. In particular, such proteins as myoglobin, ovalbumin, bovine serum albumin, and catalase, on electrophoresis with SDS, reveal bands corresponding to both monomer and dimer. The boiling of the acylase in 8 M urea containing 2% of SDS for 15 minutes that we used led to a considerable decrease in the high-molecular-weight band and to a corresponding increase in the intensity oj the band corresponding to a protein with a molecular weight of 60,000.

This gives ground for assuming that the enzyme consists of two equal subunits with a molecular weight of 60,000, but their association is so strong that it is impossible to achieve complete dissociation of the dimer. On electrophoresis the presence of SDS, acylase 1 from procine kidney shows an intense band of the band the monomer with a molecular weight of 43,000 and a weak band of the dimer with a molecular weight of 86,000 [14].

TABLE 2. Amino Acid Composition of Acylases of Different Origins

Amino acid	Aspergillus oryzae		Acylase 1 from porcine kidney [14]	
	per subunit	per 100 residues	per subunit	per 100 residues
Asp	73	13,3	31	8,2
Thr	40	7,3	23	6,0
Ser	41	7,5	24	6,3
Glu	52	9,5	48	12,5
Pro	32	5,8	27	7,1
Gly	50	9,1	31	8,2
Ala	51	9,3	35	9,2
Val	35	6,4	32	8,4
Ile	31	5,6	11	3,0
Leu	42	7,6	35	9,2
Tyr	25	4,5	10	2,6
Phe	31	5,6	21	5,4
His	11	2,0	12	3,3
Lys	16	2,9	18	4,6
Arg	19	3,4	21	5,4
Total	550	100	380	100

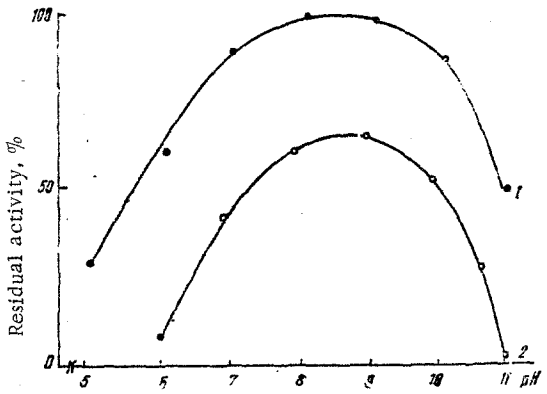

Fig. 7. Dependence of the stability of the enzyme on the pH and the time: activities of the enzyme after incubation 1) for 30 min; 2) for 100 h.

In the hydrolysis of acetylmethionine, the enzyme exhibited its maximum activity pH 8.2-9.5 (Fig. 6). The acylase is stable in the pH range of 7-10, the stability depending to considerable degree on the concentration. For example, the activity of a dilute solution (0.1 μg/ml) after 100 h at pH 8.3 at room temperature amounted to 70%, while the activity of the concentrated enzyme (0.2 mg/ml) remained unchanged for a year.

Figure 8 shows the dependence of the rate of hydrolysis of N-acetyl-L-methionine on the temperature. The temperature optimum of the activity of the enzyme is 61°C. At the same time, the acylase is unstable on heating. After 5 min at 60°C, the enzyme was 55% inactivated, and after 15 minutes 90% inactivated. However, in the presence of a substrate — N-acetyl-D,L-methionine — the acylase did not undergo inactivation at 60°C even after 30 min. Consequently, a low-molecular-weight substrate protects the enzyme from thermal inactivation.

The acylase from *Aspergillus oryzae* is a metal-dependent enzyme. EDTA in a concentration of 10^{-4} M completely inhibits its activity. When 10^{-3} M Co^{2+} was added to the enzyme that had been treated with EDTA, its activity was completely restored, while Mn^{2+}, Zn^{2+}, Sn^{2+}, and Pb^{2+} ions in the same concentrated reactivated the acylase only to the extent of 10-20%. When Co^{2+} and Zn^{2+} ions were added to the EDTA-treated enzyme, there was only 20% reactivation, which is probably connected with the competition of the metals for binding in the active center. Consequently, the acylase molecule contains cobalt or zinc ions in the active center. Ions of heavy metals — Hg^{2+}, Cd^{2+}, Cu^{2+} — in a concentration of 10^{-4} M completely inactivated the enzyme in 30 min (Table 3).

TABLE 3. Influence of Some Reagents on the Activity of the Acylase

Reagents	Concentration, M	Residual activity, %
EDTA	10^{-4}	0
p-Chloromercuribenzoate	$2 \cdot 10^{-4}$	50
	10^{-3}	0
P-Nitrobenzenediazonium chloride	10^{-5}	20
	10^{-3}	0
N-(Dimethylaminopropyl)-N'-p-phenyl-azophenylcarbodiimide methiodide	10^{-4}	70
	10^{-3}	10
Diethyl pyrocarbonate	10^{-3}	100
Tosyl-L-phenylalanine chloromethyl ketone	10^{-4}	100
p-amino-ω-bromo-m-nitroacetophenone	10^{-3}	100 (pH 6)
	10^{-3}	100 (pH 8)
Phenylmethanesulfonyl fluoride	10^{-3}	100
N-Diazoacetyl-N'-(2,4-dinitrophenyl)ethylene-diamine (in the absence of Cu^{2+})	10^{-3}	100
$CoCl_2$	10^{-5}	65
"	$5 \cdot 10^{-5} - 10^{-4}$	100
"	$5 \cdot 10^{-4}$	80
"	10^{-3}	0
without Co^{+2}	—	25
$ZnSO_4$	10^{-4}	20
"	10^{-3}	0
$CdCl_2$	10^{-4}	0
$CuSO_4$	10^{-4}	0
$Hg(CH_3COO)_2$	10^{-4}	0
$CaCl_2$	10^{-4}	100
$MnCl_2$	10^{-4}	100
$SnCl_2$	10^{-4}	100
$Pb(CH_3COO)_2$	10^{-4}	100

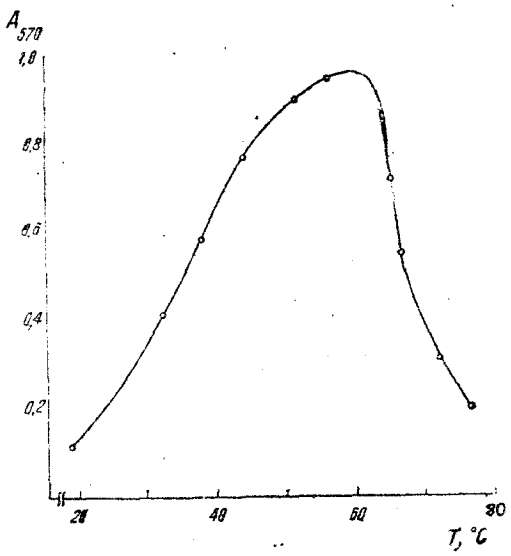

Fig. 8. Temperature dependence of the activity of the enzyme.

The enzyme that we had purified was activated by Co^{2+} ions in a concentration of 10^{-4}-$5 \cdot 10^{-5}$ M. The rate of hydrolysis of N-acetyl-L-methionine-L-serine, -L-phenylglycine, and -L-alanine in the presence of Co^{2+} ions increased by a factor of 3-4. The optimum ratio of substrate to cobalt was 1:200. The cobalt activation of acylases from fungii of the genus *Aspergillus* has been shown previously on poorly purified preparations [4, 6].

TABLE 4. Relative Rates of Hydrolysis of N-Acetyl-L-Amino Acids by Acylases

L-Amino acid giving the N-acetyl derivative	Aspergillus oryzae	Acylase 1 from porcine kidney	
		[10]	[13]
Methionine	=100	=100	=100
Leucine	128	25	40
Alanine	93	7	24
Norvaline	77	58	60
Valine	40	10	14
Tryptophan	73	0	0
Serine	40	—	—
α-Phenylglycine	11	—	—
Aspartic acid	0	—	0
Ornithine	4	—	—
α-N-Acetyllysine	0	—	—

TABLE 5. Comparison of the Molecular and Enzymatic Properties of Acylases of Different Origins

Parameters	Aspergillus oryzae	Acylase 1 from porcine kidney
Molecular weight	118 000	86 000 [14] 76 000 [13]
Isoelectric point	5,0	4,0 [14]
pH-optimum	8,0—9,5	7,0—7,8 [14]
Temperature optimum	61°	55° [14]
Activation of hydrolysis by Co^{2+} ions	Yes	No [7, 13, 14]
K_M	$15 \cdot 10^{-3}$ M	$5 \cdot 10^{-3}$ M [10] $6,6 \cdot 10^{-3}$ M [12]
Inhibition by	Residual activity, %	
diethyl pyrocarbonate 10^{-4} M	100	0 [9]
tosyl-L-phenylalanine chloromethyl ketone 10^{-4} M	100	0 [8]

To determine the groups of the enzyme that participate in enzymatic catalysis, we studied the influence of a number of reagents (Table 3). The considerable inactivation under the action of the water-soluble colored carbodiimide and of p-nitrobenzenediazonium chloride is possibly due to a modification of the carboxy groups of the residues of dicarboxylic amino acids and of tyrosine. However, the high molar ratios of enzyme to inhibitor of $1:10^3$ to $1:10^4$, do not yet permit the specificity of these reactions to be judged. The 50% inactivation of the enzyme by p-chloromercuribenzoate shows the possible presence of free SH groups essential for activity. But, as in the preceding case, the reaction cannot be considered specific because of the high concentration of the reagent ($2 \cdot 10^{-4}$ M).

Inhibitors of acylase 1 from procine kidney — tosyl-L-phenylalanine chloromethyl ketone and diethyl pyrocarbonate [8, 9] — did not affect the activity of the fungal acylase. p-Amino-ω-bromo-m-nitroacetophenone and N-diazoacetyl-N'-2,4-dinitrophenylethylenediamine did not inactivate the acylase in the absence of Cu^{2+} ions. A specific inhibitor of serine proteases — phenylmethanesulfonyl fluoride — likewise had no affect on our enzyme.

In a study of substrate specificity we determined the rate of hydrolysis of various N-acetyl-L-amino acids (Table 4). It was established that the hydrophobic amino acids are hydrolysed particularly rapidly.

The acylase from *Aspergillus oryzae* possesses an extremely weak peptidase activity. The rate of hydrolysis of such dipeptides as Gly-Ala, Gly-Leu, Ala-Ala, and Gly-Phe was 2-3 orders of magnitude lower than the rate of hydrolysis of N-acetyl-L-methionine and two orders of magnitude lower than the rate of hydrolysis of the same dipeptides by acylase 1 from porcine kidney [11, 12].

A comparison of the molecular properties (molecular weights, amino acid compositions, pI values) and functional properties (substrate specificity, inhibition, activation by cobalt, pH optimum) revealed substantial differences between the acylases (Table 5).

It is possible that N-acetyl-L-amino acids are not the best substrates of the acylases, since K_M for both enzymes for these substrates are relatively high: $5 \cdot 10^{-3}$ M and $15 \cdot 10^{-3}$ M. It may be assumed that the acylases while similarly catalysing the cleavage of a common "poor" substrate may fulfill different functions in the animal and microbial organism.

EXPERIMENTAL

The acylase was isolated from the industrial preparation "Amilorizin II 10 X", which consists of a powder of a surface culture of *Aspergillus oryzae*. The preparation had an activity in the hydrolysis of N-acetyl-L-methionine of 2.5 μmole/mg·h.

Determination of Activity. The rate of hydrolysis of N-acetyl-L-methionine was judged from the amount of methionine liberated, which was determined by the ninhydrin method.

To 0.9 ml of a 0.02 M solution of N-acetyl-D,L-methionine in 0.04 M borate buffer, pH 8.3, containing 10^{-4} M Co^{2+} ions was added 0.1 ml of enzyme solution, and the mixture was incubated at 37°C for 30 min. To determine the concentration of the methionine liberated, a sample was boiled in the water bath for 15 min with 1 ml of a 2% solution of ninhydrin in 1 M acetate buffer, pH 5.0, containing $2 \cdot 10^{-3}$ M $SnCl_2$. Then 5 ml of 60% ethanol was added and the absorption was measured at 570 nm in the 1-cm cell of a SF 16 spectrophotometer. An increase in A_{570} of one unit corresponds to 0.5 μmole of methionine in the sample.

Isolation of the Acylase. A solution of 90 g of dry "Amilorizin" powder in one liter of 0.1 M borate buffer, pH 7.0, was centrifuged at 2400 rpm at 0°C for 45 min. The supernatant, containing 40 g of protein was precipitated with ethanol cooled to −16°C, and fractions were collected in 30-50% interval. The precipitate containing the active enzyme was dissolved in 0.02 M borate buffer, pH 8.0, and was dialyzed against the same buffer for 12 h.

Chromatography on DEAE-Cellulose (Fig. 1). A solution containing 3100 A_{280} units (300 ml) was deposited on a column (2.5 × 40 cm) of DEAE-cellulose equilibrated with 0.04 M borate buffer, pH 8.0. Elution was performed with a linear concentration gradient of NaCl (0-0.5 M) in the same buffer at the rate of 40 ml/h, 12-ml fractions being collected.

Gel Filtration on Sephadex G-200 (Fig. 2). The solution (50 ml) was deposited on a column (5 × 100 cm) of Sephadex G-200 equilibrated with 0.1 M borate buffer, pH 8.3. Elution was carried out with the same buffer at the rate of 100 ml/h, 10-ml fractions being collected

Chromatography on DEAE-Cellulose at pH 5.7 (Fig. 3). The fractions containing activity (see Fig. 2) were deposited on a column containing 10 ml of DEAE-cellulose equilibrated with 0.1 M acetate buffer, pH 5.7. Elution was performed with a linear gradient of NaCl concentrations (0.1-0.5 M) in the same buffer.

Gel Filtration on Bio-Gel P-300 (Fig. 4). The enzyme solution (20 ml) was concentrated in an Amicon ultrafiltration cell with a UM-10 membrane to 5 ml and was deposited on a column (1.4 × 90 cm) of Bio-Gel P-300 equilibrated with 0.08 M borate buffer, pH 8.3. Elution was carried out with the same buffer at the rate of 12 ml/h, 3 ml fractions being collected.

Disk electrophoresis was performed in a phosphate buffer system with a separation pH of 9.0 in 7.5% polyacrylamide gel at a current strength per column of gel of 4 mA. The proteins were stained with a 0.25% solution of Coomassie Blue G-250 in 7% acetic acid.

Isoelectric focusing was performed on a Multifor instrument (LKB, Sweden) in accordance with the instructions, using a pH 3-9.5 gradient or, for more accurate determination of pI, a pH 2-5 gradient.

Electrophoresis in the presence of 0.1 SDS was performed in 5% gel by the Weber–Osborn method [15]. To intensify the dissociation of the amylase dimer, the sample of protein was boiled for 15 minutes with 8 M urea, 2% SDS, and 0.1 % mercaptoethanol.

The molecular weight of the enzyme was determined by gel filtration on a column (1.4 × 100 cm) of Sephadex G-200 and by electrophoresis in the presence of SDS in 5% polyacrylamide gel. A solution of 100 μg of the protein in 0.02 ml of 10% SDS solution containing 48 mg of urea and 0.1% mercaptoethanol was boiled in the water bath for 15 min and was then cooled and deposited on one column of the gel. As protein markers we used ovalbumin (45,000), bovine serum albumin (67,000), chymotrypsinogen A (25,000), aldolase (147,000), and catalase (240,000) (Serva, GFR).

Amino Acid Composition. The enzyme was hydrolyzed with 5.7 N HCl at 105°C for 24, 48, and 72 h and was analyzed on a Durrum D-500 amino acid analyzer (USA).

The hydrolysis of the dipeptides was carried out at pH 8.5-9.0 37°C, for 14 h, using an enzyme concentration of 1 μg/ml and a dipeptide concentration of 0.05 M. The hydrolysis products were analyzed on a BC-200 amino acid analyzer (USA).

Inhibition. The reactions of the acylase with inhibitors were carried out at 20°C for 30 min with an enzyme concentration of 0.1 μg/ml and inhibitor concentrations of 10^{-3}-10^{-4} M.

pH Stability. To 0.1-ml portions of a solution of the enzyme were added 0.2-ml portions of the following buffer mixtures, respectively: 0.1 M citric acid–Na_3PO_4 (pH 5.0-7.0); 0.1 M borate–NaOH (pH 8.0-11.0). The mixtures were kept for 20 min and 100 h, after which the activity was determined in an aliquot. The activity of a solution of the enzyme at pH 8.5 was taken as 100%.

The pH optimum was determined by performing the hydrolysis of N-acetyl-D,L-methionine in 0.1 M borate buffer, pH 7.0-9.5.

SUMMARY

A scheme is described for the isolation of highly purified L-amino acid acylase from *Aspergillus oryzae* which includes the extraction of the enzyme from the preparation "Amilorizin," fractionation with ethanol, chromatography on DEAE-cellulose, and gel filtration of Sephadex G-200 and Bio-Gel P-300. The enzyme purified 1240-fold has a molecular weight of 118,000 and apparently consists of two subunits each with a molecular weight of 60,000, and is stable in the pH interval of 7-10, its optimum pH being 8.5 and its pI 4.0. Its amino acid composition has been determined and its substrate specificity has been studied. The acylase is a metalloenzyme: Co^{2+} ions in a concentration of 10^{-4}-$5 \cdot 10^{-5}$ M increase the rate of hydrolysis of N-acetyl-L-amino acids by a factor of 3-4. Its molecular and functional properties differ from those of acylase 1 from porcine kidney.

LITERATURE CITED

1. T. Watanabe, T. Mori, and I. Chibata, Biotech. Bioeng., 21, 477 (1979).
2. E. N. Makarova, Biol. Zh. Arm., 32, No. 9, 850 (1979).
3. Y. Minematsu, Bull. Chem. Soc. Jpn., 52, No. 6, 1899 (1978).
4. K. Hirano, I. Karube, and S. Suzuki, J. Ferment. Technol., 55, No. 4, 401 (1977).
5. Y. Kameda, T. Hase, S. Kanatomo, and K. Miyazaki, Chem. Pharm. Bull., 26, No. 9, 2710 (1978).
6. I. A. Yamskov, M. V. Budanov, L. S. Lobareva, M. K. Malinka, and V. D. Davankov, Biokhimiya, 46, 651 (1981).
7. W. Kördel and Fr. Schneider, Z. Naturforsch., 32, 342 (1977).
8. Y. Frey, W. Kördel, and Fr. Schneider, Z. Naturforsch., 32, 759 (1977).
9. W. Kördel and Fr. Schneider, Z. Naturforsch., 32, 337 (1977).
10. V. K. Shvyadas, I. Yu. Galaev, N. A. Galstyan, and I. V. Berezin, Biokhimiya, 45, 1361 (1980).
11. E. Moravcsik, Y. Telegdi, H. Tüdös, K. Kömives, and L. Ötvös, Acta Biochim. Biophys. Acad. Sci. Hung., 12, No. 4, 399 (1977).
12. W. Kördel and Fr. Schneider, Z. Physiol. Chem., 356, 915 (1975).
13. F. Brubs and Ch. Schulze, Biochem. Z., 336, 162 (1962).
14. W. Kördel and Fr. Schneider, Biochim. Biophys. Acta, 445, 446 (1976).
15. K. Waber and M. Osborn, J. Biol. Chem., 224, 4406 (1969).
16. M. W. Hunkapiller and L. E. Hood, Science, 207, No. 4430, 523 (1980).