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YEAST AMINOPEPTIDASE I

CHEMICAL COMPOSITION AND CATALYTIC PROPERTIES

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Summary

An aminopeptidase (α-aminoacyl L-peptide hydrolase, EC 3.4.11.1) was purified to homogeneity from autolysates of brewer's yeast. The enzyme which is responsible for most of the yeast cell's aminopeptidase activity is a glycoprotein containing about 12% of conjugated carbohydrate and 0.02% Zn^{2+} and having a complex quaternary structure. The active species has a molecular weight of approx. 600 000 and an isoelectric point of 4.7. The enzyme is remarkably stable, even in dilute solutions. All types of L-amino acid and peptide derivatives containing a free amino terminus are attacked, including amino acid amides and esters. As to its substrate specificity, the enzyme belongs to the so called leucine-aminopeptidases. It is strongly and specifically activated by Zn^{2+} and Cl^- (or Br^-) and inactivated by metal-chelating agents. The activation by Zn^{2+} seems to be mediated by a conformational transition which affects exclusively V and leads to a form of the enzyme which enhanced stability against heat. Halide anions, on the other hand, are acting as positive allosteric effectors, modulating both V and K_m.

Introduction

Since the pioneering work of Grassmann and his coworkers [1—5] on the proteolytic enzymes of yeast in the late twenties and early thirties, some of the enzymes responsible for the activities differentiated in these studies have been purified and characterized in considerable detail. Thus, three different proteinases were isolated and studies are now in progress in several laboratories. Recently a metal-dependent dipeptidase acting on a variety of dipeptides was obtained in homogeneous form [6]. However, the number and properties of aminopeptidases from yeast remains unclear, since the substrate specificity of Grassmann's preparation [4] seems to differ from a more thoroughly purified

enzyme described by Johnson in 1941 [7] and a preparation studied by Cordonnier [8]. In the present paper evidence is furnished indicating that almost all of the yeast cell aminopeptidase activity is due to only one enzyme; the purification and some properties of this enzyme are described.

Materials

Inorganic chemicals were of reagent grade by Merck, Darmstadt unless indicated otherwise. Most of the substrate peptides, including Leu-Gly-Gly the usual test substrate, were purchased from Fluka, Buchs, some others from Serva, Heidelberg and used without further purification. Ala-Asp-Phe-OMe, Val-Ala-Thr-Ala, Ala-Thr-Ala, Ala-Thr-Gly and the corresponding methyl esters as well as Thr-Ala were prepared by Drs. E. Schaich and A. Raschig in our laboratory [9]. Ala-Gly-Gly ethyl ester, Val-Gly and Thr-Gly were synthetized according to published procedures. In most cases the purity of the substrates was assessed by C, H, N- and amino acid analysis. DEAE-cellulose SH and the chemicals used for the electrophoreses as well as bovine serum albumin, hemoglobin and catalase were products of Serva, Heidelberg. Lactate dehydrogenase and β-galactosidase were obtained from Boehringer, Mannheim. Sephadex and Sepharose gels were purchased from Deutsche Pharmacia, Frankfurt.

Methods

Aminopeptidase assay. (a) The hydrolysis of amino acid *p*-nitroanilides was assayed according to Tuppy et al. [10]. This method was routinely used to monitor aminopeptidase activities during purification.

(b) The hydrolysis of dipeptides and those higher peptides yielding virtually inert dipeptide products (e.g. of tripeptides of the type X-Gly-Gly) was followed spectrophotometrically according to Schmitt and Siebert [11]. Usually 50 µl of enzyme solution was preincubated in semi-micro silica cells for 10 min at 40°C with 500 µl of Kolthoff phosphate/borate buffer, pH 7.8 (made up by titrating 0.1 M KH_2PO_4 with 0.05 M borax solution to the desired pH) containing 100 mM NaCl and 0.1 mM Zn^{2+} (both ions are required for full activity: see below). Then the reaction was started by addition of 100 µl of prewarmed substrate solution of 6.5 times the final concentration. Initial velocities were derived from the absorbance change with time at 235 nm as measured in a Zeiss PMQ II spectrophotometer.

(c) Substrates not suitable for test (b) were incubated with enzyme in appropriate buffers (usually in the buffer system specified above). Samples were removed at various times and the reaction terminated by mixing with excess 0.2 M citrate/HCl buffer, pH 2.2. Then the samples were subjected to amino acid analysis, the procedure adopted depending on the separation to be effected. In most cases total analysis times of 40—50 min on a 0.9 × 15 cm column of Beckman M 72 resin were sufficient to separate the species of interest.

One unit of aminopeptidase activity is defined as the amount of enzyme splitting off 1 µmol of amino-terminal amino acid per min.

Protein determinations. Routinely, protein concentrations were determined from the A_{280nm}/A_{260nm} ratio according to Warburg and Christian [13] some-

times by the micro-biuret assay [14]. The A_{280nm} of a 0.1% solution of the pure enzyme was 0.85 at neutral pH at a A_{280nm}/A_{260nm} ratio of 1.7.

Carbohydrate determinations. Neutral sugars, hexosamines and sialic acids were assayed separately after acid hydrolysis and separation of the different carbohydrate fractions on ionic exchange columns (see ref. 15). Total neutral hexoses were determined by the Anthron reaction [15], hexosamines by the Elson-Morgan reaction as modified by Gatt and Berman [16]. Sialic acid determinations were performed by the resorcinol method [17], free glucose was measured enzymatically with glucose oxidase/peroxidase.

Zinc determination. Zinc was measured by atomic absorption spectrophotometry with a Perkin-Elmer 300 SG photometer equipped with graphite cell accessory HGA 70.

Amino acid analysis. Amino acid analyses were performed by standard procedures [18,19] with a Beckman Multichrom B liquid column chromatograph. Usually protein samples were hydrolyzed in 6 M HCl at 110° C after thorough flushing with argon. For the determination of tryptophan 5% thrioglycolic acid was added to the hydrolysis mixtures [20]. Cysteine (as cysteic acid) and methionine (as methionine sulfone) were assayed after performic acid oxidation [21].

Electrophoretic procedures. Polyacrylamide disc electrophoreses were run in 5% gels with 0.2% methylene-bis-acrylamide as cross-linking agent at pH 8.9. The composition of gels and buffers was as described by Maurer ("system I") [22]. Sodium dodecyl sulfate electrophoreses were carried out with 7.5% gels according to Weber and Osborn [23]. β-Galactosidase (*Escherichia coli*), phosphorylase A (rabbit muscle), catalase (bovine spleen), lactate dehydrogenase, chymotrypsinogen and lysozyme were used as reference proteins.

Density gradient centrifugations Density gradient centrifugations were performed according to Martin and Ames [24] using 5—20% (w/w) sucrose density gradients at 4° C in the SW 50 L rotor of a Spinco L2 ultracentrifuge. β-Galactosidase (*E. coli*), catalase and asparaginase (*E. coli*) were applied as reference proteins.

Purification of the enzyme. Source of the enzyme was brewer's yeast obtained from a local brewery. Prior to use the cells could be stored frozen for months without any great loss of aminopeptidase activity. With the exception of autolysis all steps specified below were performed at 0—4° C usually starting with 3—5 kg of pressed yeast. Because steps 1—4 of the purification are the same as described previously for the purification of yeast dipeptidase [6] they are only briefly outlined.

(1) Autolysis: 36—72 h in 0.2 M ammonium acetate buffer, pH 6.2, containing 1 mM Zn^{2+} at room temperature.

(2) $(NH_4)_2SO_4$ fractionation: The protein fraction precipitating between 50 and 70% saturation is collected and dissolved in the minimum amount of the buffer already used for autolysis.

(3) Heat precipitation: Heating for 2 min to 60° C and rapid cooling. Remove precipitated protein by centrifugation.

(4) Chromatography on Sephadex G-150: Performed on the supernatant of step 3 using a 10 × 100 cm Sephadex G-150 column equilibrated with 0.01 M Tris/succinate buffer, pH 7.3, containing 0.1 mM Zn^{2+} (Buffer A).

(5) Acetone precipitation: The active fractions from the preceding step are pooled and brought to pH 5.8 by addition of acetic acid. Then acetone precooled to −15°C is added slowly to 30% of volume. After some standing the precipitate is collected by centrifugation (20 min at 10 000 × *g*) and dissolved in the minimum amount of Buffer A.

(6) DEAE-cellulose chromatography: The enzyme solution from the preceding step is applied to a 2 × 40 cm DEAE-cellulose column. The column is washed with Buffer A until no further protein is emerging. The aminopeptidase is then eluted within 600 ml of a linear NaCl gradient (0—500 mM) in Buffer A.

(7) Rechromatography on DEAE-cellulose: The active fractions from step 6 are pooled and diluted to a NaCl concentration of 80 mM (checked by conductivity measurements). The enzyme is then adsorbed to a 3 × 10 cm DEAE-cellulose column and eluted with a concave NaCl gradient (80—300 mM) in Buffer A. Then the enzyme solution is concentrated to a volume of 5 ml or less in an Amicon ultrafiltration cell (membrane PM 10).

(8) Gel filtration on Sepharose 6B: Finally the aminopeptidase solution from step 7 is applied to a 2 × 50 cm Sepharose 6B column equilibrated with and eluted with 50 mM ammonium acetate buffer, pH 6.2. The enzyme emerging from this column may be stored frozen without further treatment or isolated by acetone or $(NH_4)_2SO_4$ precipitation.

Results

Purification of the enzyme

Data illustrating a typical purification procedure are summarized in Table I. The two steps following autolysis remove the bulk of inactive protein and leave

TABLE I

PURIFICATION OF YEAST AMINOPEPTIDASE I

Purification was carried out as described in Methods. The data given refer to a starting amount of 1 kg of pressed yeast. Activities were measured with 30 mM Leu-Gly-Gly as a substrate using assay (b).

Step	Protein (mg)	Total activity (units)	Spec. activity (units/mg)	Yield (%)	Purification
(1) Autolysate (72 h)	44 000	36 000	0.83	100	1
(2) $(NH_4)_2SO_4$ precipitation	14 300	25 000	1.75	69	2.1
(3) Heat precipitation	10 500	24 000	2.3	66	2.8
(4) Sephadex G-150 chromatography	336	17 000	50.5	47	61
(5) Acetone precipitation	275	15 300	56	42	68
(6) DEAE-cellulose chromatography	67	13 000	194	36	234
(7) DEAE-cellulose rechromatography	25	11 300	452	31	545
(8) Sepharose 6B chromatography	10	7 650	765	21	922

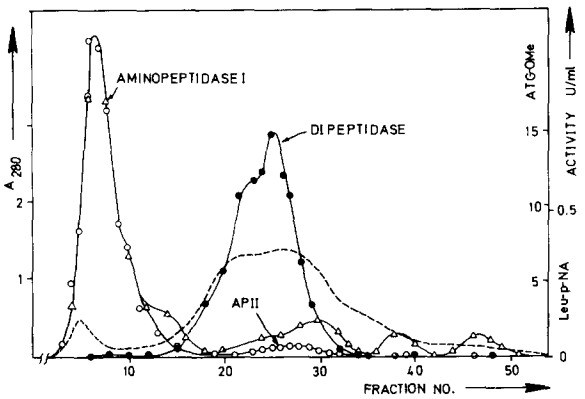

Fig. 1. Chromatography of a crude yeast peptidase preparation on Sephadex G-150. A sample of the 50—70% $(NH_4)_2SO_4$ precipitate (about 120 mg of protein) was dissolved in 0.05 M Tris · HCl buffer, pH 7.3, and subjected to gel filtration on a 2.5 × 90 cm Sephadex G-150 column, equilibrated and eluted with the same buffer. The absorbance at 280 nm (- - - - - -) and the activities toward leucine *p*-nitroanilide (△), L-Ala-L-Thr-Gly-OMe (○) and Gly-L-Leu (●) were measured as described in Methods with the eluted fractions (2.5 ml each). The column dimensions and the buffer applied in this analytical run are not the same as routinely used in the large-scale preparation of the enzyme.

both dipeptidase and aminopeptidase in a considerably enriched state. The subsequent gel filtration (see Fig. 1) quantitatively separates aminopeptidase I from dipeptidase and a much smaller peak of aminopeptidase activity. This enzyme (aminopeptidase II) differs from the high molecular weight aminopeptidase I with respect to its substrate specificity (see Table IV). No attempts have been made to characterize this enzyme in detail; it seems likely, however, that it is identical to an enzyme very recently described by Masuda et al. [25] and called aminopeptidase I by these authors.

The high molecular weight enzyme I is obtained in a homogeneous form by two successive ion-exchange chromatography steps on DEAE-cellulose following the Sephadex G-150 step and an acetone precipitation. During rechromatography the enzyme is separated into several species with similar elution behavior and nearly identical specific activities (Fig. 2). The final gel filtration on Sepharose 6B, shown in Fig. 3, results in a further increase of specific activity even if performed with a pure enzyme preparation because in dilute solutions pH-dependent equilibria between active aminopeptidase I (which has a molecular weight of about $6 \cdot 10^5$, see below) and inactive subfragments as well as higher aggregates are established. As will be shown elsewhere (Metz, G. and Röhm, K.-H., unpublished) all the protein species shown in Fig. 3 have very similar amino acid composition and are mutually interconvertible.

At the final stage of purification specific activities of up to 980 units/mg were observed as measured with 30 mM Leu-Gly-Gly at pH 7.8 in the presence of 100 mM Cl^- and 0.1 mM Zn^{2+}. With Ala-Thr-Ala, the best substrate found thus far, values of more than 3000 units/mg were found which correspond to a turnover number of about $2 \cdot 10^6$ min^{-1} (calculated from one active site per 600 000 daltons).

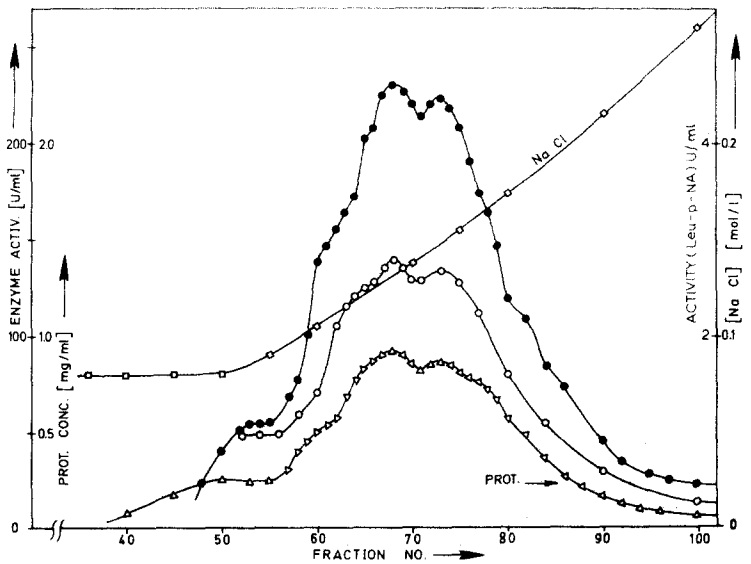

Fig. 2. Rechromatography of aminopeptidase I on DEAE-cellulose. Rechromatography on a 3 X 10 cm DEAE-cellulose column was performed with about 160 mg of enzyme as described in Methods. A concave gradient from 80 to 300 mM NaCl was applied to elute the enzyme. The NaCl concentrations (□) and protein contents (△, determined by the micro-biuret assay) of the eluted fractions (4 ml each) as well as the activities against Leu-Gly-Gly (●) and Leucine *p*-nitroanilide (○) are shown.

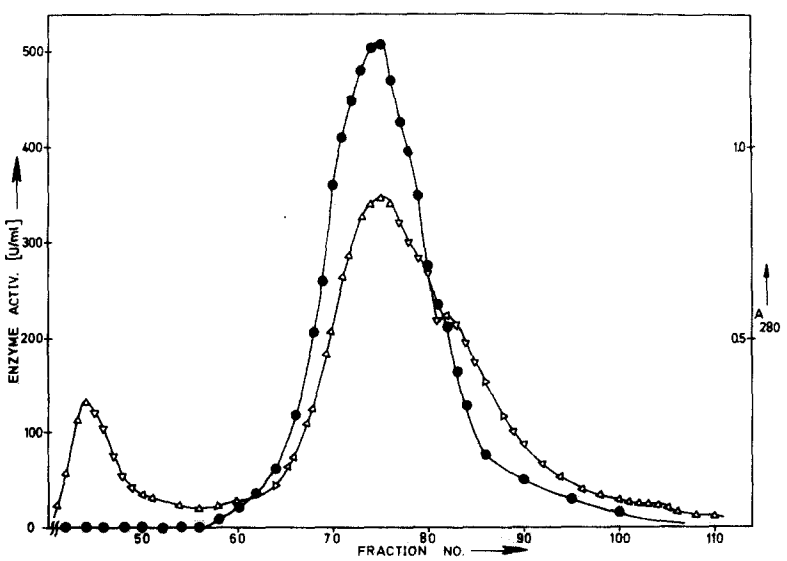

Fig. 3. Gel filtration of purified aminopeptidase I one Sepharose 6B. The pooled active fractions from a DEAE-cellulose rechromatography (about 60 mg of protein) were applied to a 2 X 50 cm Sepharose 6B column and eluted with 50 mM ammonium acetate buffer, pH 6.2. The absorbance at 280 nm (△) and the activities towards Leu-Gly-Gly (●) are plotted vs. the fraction number.

Alternative purification methods

If the recovery of dipeptidase is not of interest, the initial steps of the above method may be replaced by the procedure of Grassmann et al. [4] which includes an isoelectric precipitation at pH 4.7, followed by sodium acetate and acetone precipitation steps. A considerable purification (up to 300 units/mg) may be accomplished by this method. However, the yields of the acid precipitation steps varied considerably with different preparations and were rather poor in some cases. Moreover, yeast dipeptidase is completely inactivated at pH 4.7. Purification to homogeneity may then be achieved by steps 6—8 of our procedure. Thereby, we found that the aminopeptidase we isolated by Grassmann's procedure [4] was identical with the enzyme resulting from our own purification method, not only with regard to its molecular weight but also in its substrate specificity (see Table IV). Attempts to obtain homogeneous enzyme by the method of Johnson [7] remained unsuccessful.

Criteria of purity

If isolated by acetone precipitation prior to the run, the purified enzyme shows a single main band in disc electrophoresis, accompanied by several faint ones which are due to subfragments of the active enzyme. The aminopeptidase activity visualized by activity staining with leucine β-naphthylamide [12] comigrates with the main protein band (see Fig. 4). With not acetone-precipitated samples this electrophoretic polymorphism, which will be discussed in more detail elsewhere (Metz, G. and Röhm, K.-H., unpublished), is much more pronounced. Only one major band, however, appears in sodium dodecyl sulfate electrophoresis having a molecular weight of 51 000 ± 2000, a small peak corresponding to the dimer of this species may be observed in addition.

Molecular weight

The catalytically active species of yeast aminopeptidase I sediments in sucrose density gradients with a sedimentation constant of 19—20 S. This is in satisfactory agreement with data of Johnson [7] who calculated a molecular weight of 670 000 from $S^0_{20,w} = 21$ S and a diffusion constant of $3.1 \cdot 10^{-7}$ cm^2/s without giving a value for the partial specific volume, however. From the amino acid composition and the carbohydrate content of our enzyme (see below) a value of $v = 0.725$ cm^3/g may be estimated [26,27]. Using this value together with the diffusion constant reported by Johnson [7] we calculate a molecular weight between 560 000 and 615 000. As mentioned above, the main species found in the sodium dodecyl sulfate electrophoresis has a molecular weight of $5.1 \cdot 10^4$. Since there is evidence indicating the native enzyme consists of 12 sodium dodecyl sulfate subunits (Metz, G. and Röhm, K.-H., unpublished) we may calculate a molecular weight of $6.1 \cdot 10^5$, a result which is in satisfactory accord with the ultracentrifuge data.

Isoelectric point

An isoelectric point of 4.7 was determined by isoelectric focusing (in 1% Ampholine gradients, pH 4—6). This accounts for the enzymes' minimal solubility at this pH which has been noticed by Grassmann et al. [4] as well as by Johnson [7]. Since aminopeptidase I is strongly inhibited by the carrier am-

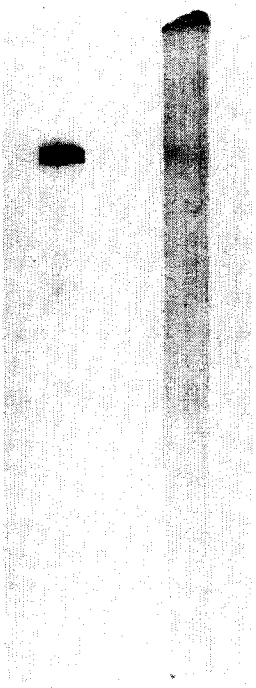

Fig. 4. Disc-electrophoresis of purified aminopeptidase I. Acetone-precipitated samples of the purified enzyme were subjected to disc-electrophoresis in 5% polyacrylamide gels at pH 9 (for details see Methods). The direction of migration was toward the anode (below). The gel shown on the left was stained for protein with Coomassie brilliant blue. On the right an activity stain with leucine β-naphthylamide [12] is shown.

pholyte the fractions from the column had to be extensively dialyzed before the assay.

Amino acid composition

The amino acid composition of the enzyme is given in Table II. The samples used were derived from the pooled active fractions of a DEAE-cellulose rechromatography (see Fig. 2). From the data of Table III a minimal molecular weight of the enzyme protein of approx. 22 000 may be estimated (based on one tryptophan per chain). By doubling this value and taking the carbohydrate content into account, a subunit M_r of about 50 000 results, which is in good agreement with the molecular weight found by dodecyl sulfate electrophoresis.

The best integer values listed in Table III have been calculated on the basis of 22 phenylalanines/subunit since the lowest variance among different determinations was found with this amino acid.

Carbohydrate content

The glycoprotein nature of the enzyme is readily demonstrated by treating polyacrylamide gels with periodate followed by Schiff's reagent [28]. The car-

TABLE II

AMINO ACID COMPOSITION OF YEAST AMINOPEPTIDASE I

If not specified otherwise mean values from 5—6 determinations are given. The amounts of the individual amino acid residues and their standard deviations are expressed as percent of the sum of all residues (g/100 g of protein). The numbers of residues were calculated assuming 22.00 phenylalanines to be present per subunit (see text).

Amino acid	Protein g/100 g ± S.D.	Nos. of residues per 22 phenylalanines	Best integer
Aspartic acid	11.57 ± 0.58	45.1 ± 2.2	45
Threonine *	5.11 ± 0.09	22.7 ± 0.4	23
Serine *	5.61 ± 0.16	28.9 ± 0.8	29
Glutamic acid	10.24 ± 0.25	35.6 ± 0.6	36
Proline *	4.46 ± 0.22	20.6 ± 1.0	21
Glycine	4.82 ± 0.09	37.9 ± 0.7	38
Alanine	4.45 ± 0.08	28.1 ± 0.5	28
Cysteine **	1.16 ± 0.08	3.9 ± 0.3	4
Valine	5.55 ± 0.35	25.1 ± 1.5	25
Methionine **	1.52 ± 0.3	5.2 ± 0.8	5(6)
Isoleucine	5.96 ± 0.20	20.4 ± 0.8	21(20)
Leucine	9.84 ± 0.24	39.0 ± 0.9	39
Tyrosine	5.46 ± 0.54	15.0 ± 1.4	15
Phenylalanine	7.22 ± 0.07	22	22
Histidine	3.39 ± 0.07	11.1 ± 0.3	11
Lysine	7.69 ± 0.10	27.3 ± 0.3	27
Arginine	5.08 ± 0.07	14.6 ± 0.2	15
Tryptophan ***	0.87 ± 0.08	2.1 ± 0.2	2
Sum	100.00		406

Molecular weight (without carbohydrates): 44 800

* Extrapolated to zero hydrolysis time.

** Two determinations, after performic acid oxydation.

*** Three determinations, hydrolyzed with 5% thioglycolic acid, no corrections made.

TABLE III

KINETIC PARAMETERS OF Ala-Thr-Ala-OMe HYDROLYSIS IN THE PRESENCE OF ACTIVATORS

The kinetic constants were derived from experiments carried out as described in the legend of Fig. 7 with initial substrate concentrations from 1 to 20 mM. Maximal velocities V (μmol/min), apparent Michaelis constants K_{app} (mmol/l) and Hill coefficients n were calculated with the aid of a FORTRAN computer program which performs a weighted fit of the Hill equation $v = V \cdot [S]^n / (K_{app} + [S]^n)$ to the velocity data.

Activator concentration		0 mM Br^-	0.4 mM Br^-	40 mM Br^-
0 μM Zn^{2+}	V	6.5	9.6	18
	K_{app}	18.5	2.4	2.0
	n	1.71	1.12	1.05
1 μM Zn^{2+}	V	22	38	48
	K_{app}	17.5	4.5	2.6
	n	1.58	0.94	0.95
100 μM Zn^{2+}	V	37	50	54
	K_{app}	25.3	5.0	3.0
	n	1.73	1.02	1.03

bohydrate bands visualized by this procedure comigrate with the bands revealed by protein staining.

Some preliminary carbohydrate determinations have been carried out as described in Methods. Since we did not separate the individual sugars, but rather groups of carbohydrates, only approximate values may be given. Standard curves were constructed with 1 : 1 mixtures of galactose and mannose (glucose was not detected in this fraction) for neutral hexoses with glucosamine for the hexosamine assay and with *N*-acetyl neuraminic acid, respectively, for the sialic acid determination.

In this way we found the enzyme to contain about 12% of weight of neutral hexoses and 0.5% of hexosamines. Sialic acids were not detected in the hydrolysates.

Zinc content

Enzyme samples which had been purified in the absence of zinc and dialyzed against several changes of dithizone-extracted ammonium acetate buffer at pH 6.5 prior to the determinations, contained 0.02 ± 0.005% by weight of zinc (corrected for the zinc content of the buffer). This is equivalent to 1.8 ± 0.5 mol of zinc/600 000 g of enzyme. The zinc concentration in the dialysis buffer, determined at the same time, was about 0.04 μM. No irreversible denaturation seems to have occurred during the preparation of the samples, since immediately after addition of Zn^{2+} and/or Cl^- normal specific activities were measured. Higher zinc contents (0.2—0.3%) were determined with an enzyme preparation purified in the presence of zinc as described in Methods.

Stability

As compared to yeast dipeptidase [6] the enzyme is remarkably stable. Solutions diluted with various buffers from pH 5 to 11 to a final concentration of about 0.1 mg/ml and kept for 3 weeks at 4°C lost in the average only about 10% of their initial activity. Repeated freezing and thawing of neutral solutions hardly affects their activities. Aminopeptidase solutions may even be concentrated in a rotary evaporator at 30°C without much loss of active enzyme.

Catalytic properties

Activation by Zn^{2+} and halide ions. It was known for a long time that yeast aminopeptidase is strongly activated by Cl^- and Zn^{2+} [7]; both activators are not uncommon with peptidases. A complete loss of activity in the presence of metal chelating agents, demonstrated by Fig. 5, shows that the enzyme's metal requirement is absolute. The efficiency of the various chelators follows essentially the same order as do the effective stability constants of their complexes with Zn^{2+} and related metals at pH 7.8. From a series of divalent metal ions (Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+} and Mg^{2+}) only Zn^{2+} was able to restore normal activities after treatment of the enzyme with EDTA and removal of the chelating agent on Sephadex G-25. Thus the enzyme exhibits a narrow specificity with respect to the activating metal. The dependence of its fractional activity on the level of free Zn^{2+}, however, suggests the existence of at least two different classes of Zn^{2+} binding sites with greatly differing affinities. This is depicted in Fig. 6 which also shows the effect of Br^- which is nearly as effective an activator as Cl^-.

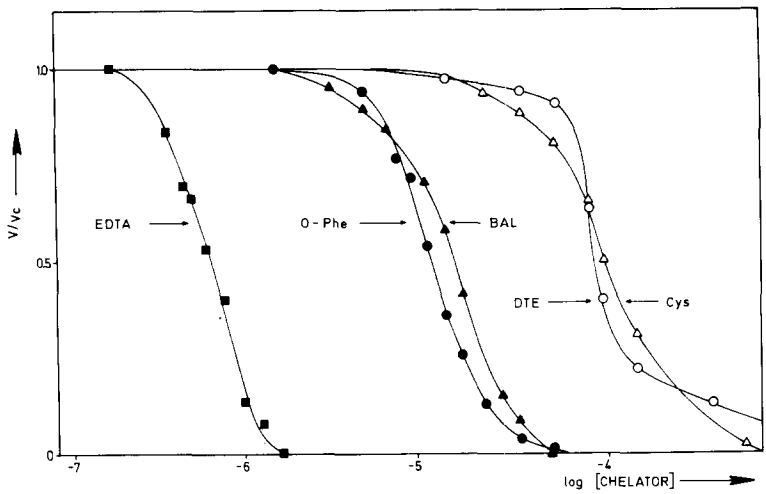

Fig. 5. Inactivation of aminopeptidase I by metal chelators. Solutions of the enzyme in phosphate/borate buffer, pH 7.8 (made up as specified in Methods) were preincubated with various concentrations of the chelating agent for 15 min at 40°C. Then substrate (leu-Gly-Gly) was added to a final concentration of 20 mM and initial velocitites were determined immediately. The ratios of the velocities measured after treatment with chelating agent and the velocities of controls (v/v_c) are plotted vs. the logarithm of the chelator concentration. The chelating agents were ethylenediamine tetraacetate (■), *o*-phenanthroline (●), dithiothreitol (○), 2,3-dimercaptopropanol (▲) and cysteine (△).

Enzyme samples freed from EDTA by passage over Sephadex columns, containing about 0.05 μM Zn^{2+}, exhibit a small residual activity which can be considerably enhanced not only by addition of Zn^{2+} (half-maximal activation was reached at about 2 μM Zn^{2+}) but may be brought to nearly the same final activity by Br^- alone (half-maximal activation at 3 mM). This cannot be due to traces of metal in the KBr used which contained less than 0.1 ppm Zn^{2+}. Thus, even at zinc concentrations far below those needed for activation the enzyme seems to contain essential Zn^{2+}. By lowering the levels of free Zn^{2+} down to 10^{-11} M using Zn^{2+}/nitrilotriacetic acid metal buffers we were able to estimate the binding constants for these more tightly bound Zn^{2+}. As shown in the insert of Fig. 6 the Zn^{2+} concentration needed for half saturation of the tighter-binding sites is by about three orders of magnitude lower than the concentrations required for activation. These results are in agreement with the finding that the enzyme still contains about 2 g atoms of zinc/mol of active enzyme at very low levels of extrinsic Zn^{2+} (see above).

Some information on the mechanisms of activation exerted by Zn^{2+} as well as Br^- (or Cl^-) is provided by their effects on the kinetics of the enzyme (see Fig. 7 and Table III). Clearly at pH 7.8 and in the absence of halide ions non-hyperbolic kinetics prevail with a good peptide substrate. The Hill coefficient n as a measure of sigmoidicity is about 1.7 under these conditions. An increase of the Zn^{2+} concentrations results in an increase of the maximal velocity V whereas K_{app} and n are not significantly affected. Such a behaviour suggests independent binding of activator and substrate to the enzyme in a random order [30]. Br^-, on the other hand, acts as a positive allosteric effector, restoring

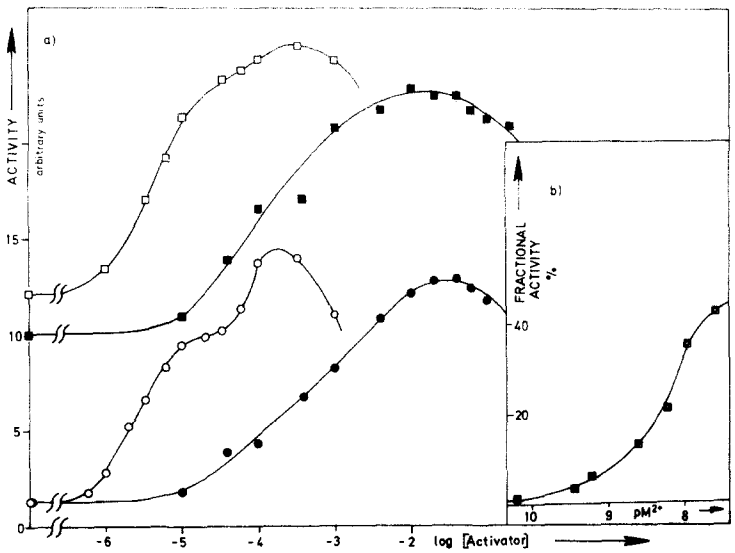

Fig. 6. Reactivation of aminopeptidase after EDTA treatment. In a) Zn^{2+} concentrations are given in terms of total zinc, the data of b were obtained using zinc/nitrilotriacetic acid metal buffers to establish sufficiently low Zn^{2+} concentrations. The third pK_a of nitrilotriacetic acid and the stability constant of its zinc complex used to calculate free zinc levels at pH 7.8 were taken from Cohen and Wilson [29]. (a) Enzyme samples were dialyzed overnight against 1 mM EDTA in 0.2 M ammonium acetate buffer, pH 6.0, and freed from the chelating agent by passage over Sephadex G-25 equilibrated with the same buffer. 100 µl of the resulting enzyme solution was then preincubated for 15 min at 40°C with 700 µl of the standard phosphate/borate buffer, pH 7.8, (see Methods) containing the amounts of activators required to yield the final concentrations given in the graph. Then the reaction was started by addition of 200 µl of 100 mM Leu-Gly-Gly in the same buffer. Initial velocities are shown determined after preincubation with (1) increasing amounts of Zn^{2+} in the absence (○) and in the presence of 100 mM Br^- (□); and (2) increasing amounts of Br^- in the absence (●) and in the presence of 50 µM Zn^{2+} (■). They are plotted versus the logarithms of activator concentrations. (b) EDTA-treated enzyme (see above) was preincubated (at 25°C overnight) with standard phosphate/borate buffer, pH 7.8, containing 100 mM Br^-, 100 mM nitrilotriacetic acid and the amount of zinc acetate required to yield the desired levels of free Zn^{2+}. Activities determined with leucine *p*-nitroanilide are expressed as percent of the value measured in the presence of 100 mM Br^- and 100 µM Zn^{2+}. They are plotted versus the negative logarithms of free Zn^{2+} concentrations (pM^{2+}).

Michaelis-Menten kinetics even if present in low amounts.

The differences between the action of both activators are also reflected by their influence on the enzyme conformation as monitored by its heat stability. So the presence of 100 µM Zn^{2+} results in a markedly enhanced heat stability, especially at high pH, whereas the residual activities after heating with Br^- are not significantly higher than those found after heat treatment without any additions.

Divergent behaviour of dipeptide hydrolysis and tripeptide hydrolysis with respect to the activation by Zn^{2+} and Cl^- which was the cause of some controversy in the early literature on yeast aminopeptidase [5,7] could not be found with our preparation.

pH dependence of the reaction

As reported earlier [5,7] the pH optimum of yeast aminopeptidase activity

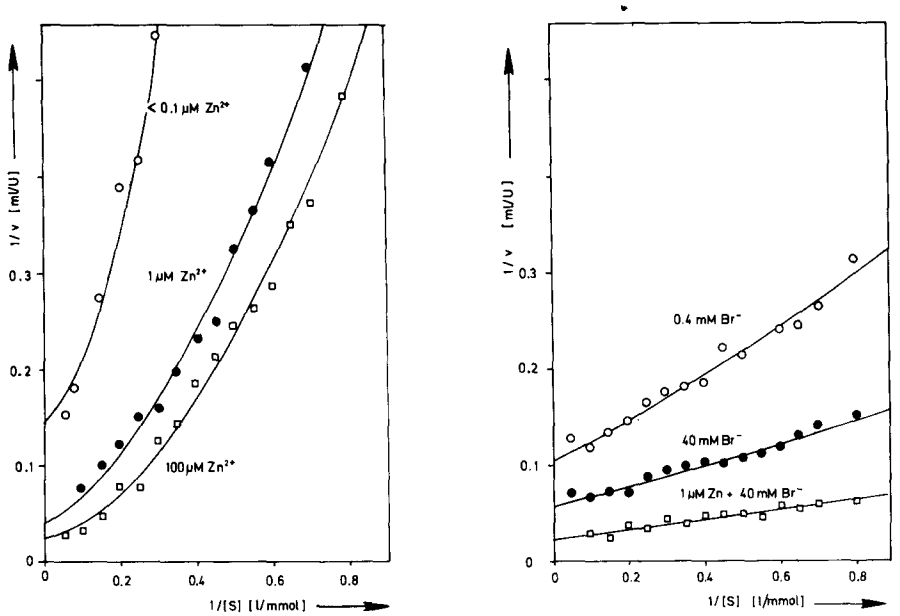

Fig. 7. The effects of Zn^{2+} and Br^- on the kinetics of Ala-Thr-Ala-OMe hydrolysis. Initial velocities were determined with enzyme solutions (EDTA-treated as described in the legend to Fig. 6a) using L-Ala-L-Thr-L-Ala-OMe as a substrate. The following concentrations of activators were applied: (a) No Zn^{2+} and no Br^- added (○); 1 μM Zn^{2+}, 0 mM Br^- (●); 100 μM Zn^{2+}, 0 mM Br^- (□); (b) 0.4 mM Br^-, 0 μM Zn^{2+} (○); 40 mM Br^-, 0 μM Zn^{2+} (●); 40 mM Br^-, 1 μM Zn^{2+} (□). Double reciprocal plots of the data are shown. The solid lines were calculated from computer-generated best fits of the Hill equation to the points (see Table III).

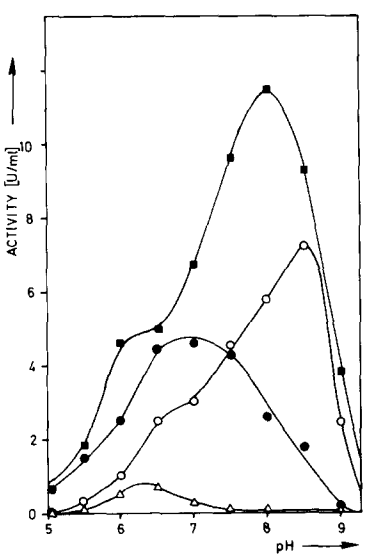

Fig. 8. The effects of activators on the enzyme's pH dependence. Enzyme samples, pretreated as described in the legend to Fig. 6a, were incubated with the indicated amounts of activators in 0.1 M succinate/borate buffers (pH 5.0 and 5.5) or phosphate/borate buffers (pH 6—9) overnight at 4° C. Then the samples were equilibrated at 40° C for 20 min and activities were measured as usual with 20 mM Leu-Gly-Gly as substrate. Velocities determined in this way with (1) no Zn^{2+} and no Br^- added (△), (2) 100 μM Zn^{2+} (●), (3) 60 mM Br^- (○) and (4) 100 μM^{2+} + 60 mM Br^- (■), are plotted versus the pH of preincubation and assay.

is located between pH 7 and 8.5 (depending on the substrate used) if Zn^{2+} and Cl^- are present. A more detailed analysis shows, however, that these pH vs. activity profiles are not likely to give the pH dependence of catalysis itself but rather the pH dependence of activation by metal and halide. This is seen from Fig. 8; clearly, the pH optimum is found between pH 6 and 6.5 if activators are absent. Another feature which modulates the observed pH vs. activity profile is the existence of association/dissociation equilibria between the active enzyme and other forms. These effects are especially important below pH 5.5 and above pH 9 (Metz, G. and Röhm, K.-H., unpublished).

Substrate specificity

Some illustrative data on the substrate specificity of the yeast enzyme are summarized in Tables IV and V. Relative velocities determined with partially purified aminopeptidase II (see Fig. 1) and with a preparation obtained by the method of Grassmann et al. [4] are included for comparison.

It is obvious that aminopeptidase I belongs to the so-called leucine aminopeptidases [31]. All kinds of amino acid and peptide derivatives are attacked

TABLE IV

SUBSTRATE SPECIFICITY OF YEAST AMINOPEPTIDASE PREPARATIONS

The substrates were normally assayed at an initial concentration of 10 mM in 0.1 M sodium-phosphate buffer, pH 7.8, containing 100 mM NaCl and 0.1 mM Zn^{2+} at 40°C. The velocities given are expressed relative to the rate of Leu-Gly-Gly hydrolysis as 100. Abbreviations: X-NH_2, amino acid amides; X-OMe, peptide methyl esters; X-p-NA, amino acid *p*-nitroanilides.

Substrate	Relative activity		
	Aminopeptidose I	aminopeptidase I (G) *	aminopeptidase II **
L-Leu-Gly-Gly	100	100	100
L-Phe-Gly-Gly	89	—	—
L-Val-Gly-Gly	3	—	—
L-Ala-Gly-Gly	91	91	9
Gly-Gly-Gly	7	—	—
Pro-Gly-Gly	0		
L-Leu-Gly	14.4	13.5	—
L-Leu-NH_2	8.1	7.9	—
L-Leu-OMe	10.2	—	—
L-Leu-β-Ala	6.9	—	—
L-Phe-Gly	23	—	—
L-Phe-NH_2	6.5	—	—
L-Phe-OMe	11	—	—
L-Ala-L-Thr-L-Ala	509	—	—
L-Ala-L-Thr-L-Ala-OMe	336	369	59
L-Ala-L-Thr-Gly-OMe	418	432	80
L-Ala-L-Asp-L-Phe-OMe	134	—	—
L-Leu-p-NA ***	1.3	—	—
L-Lys-p-NA ***	0.02	—	—
Gly-p-NA ***	0.05	—	—

* Measured with a preparation purified according to Grassmann et al. [4].

** Measured with aminopeptidase II, see Fig. 1.

*** 2 mM.

TABLE V

HYDROLYSIS OF DIPEPTIDES BY AMINOPEPTIDASE I

The substrates were assayed at concentrations of 40 mM. Initial velocities were determined with the amino acid analyzer as described in Methods. They are expressed relative to the rate of Leu-Gly hydrolysis as 100.

Substrate	Relative activity	Substrate	Relative activity
Gly-Gly	1.0	Pro-Gly	0
Gly-L-Ala	5.5	Val-Gly	<0.1
Gly-L-Leu	2.1	Thr-Ala	<0.1
L-Ala-Gly	19.7	Ala-Thr	36.5
L-Ala-L-Leu	49.4		
L-Leu-Gly	100		
L-Leu-L-Ala	155		
L-Leu-L-Leu	116		

provided they contain a free α-amino group, including amino acid amides and esters. As a rule, compounds bearing leucine or another hydrophobic amino acid in the amino-terminal position are among the best substrates whereas peptides with glycine or charged amino acid side chains in this place are split much more slowly (Compare, for instance, Leu-Gly-Gly with Gly-Gly-Gly and Leu-Gly with Gly-Gly). Amino acid *p*-nitroanilides, although conveniently assayed, are rather poor substrates.

Kinetic parameters for the hydrolysis of some representative substrates are given in Table VI. They illustrate the marked influence exerted by the substrate side chains on the kinetic behaviour of the enzyme. This is especially obvious with threonine and valine, the effect of which depends on whether they occupy the first or second position. Possible reasons for this behaviour have been discussed in detail elsewhere [32].

TABLE VI

KINETIC OF PEPTIDE HYDROLYSIS BY YEAST AMINOPEPTIDASE I

The kinetic constants of some representative di- and tripeptides were determined with 0.1 M phosphate/borate buffer, pH 7.8, containing 0.1 M NaCl and 0.05 mM Zn^{2+} at 40° C.

Substrate	V *	K (mM)	Substrate	V *	K (mM)
Ala-Gly	1.5	40	Leu-Gly	13	23
Ala-Gly-Gly	100	12	Leu-Ala	15	7
Ala-Gly-Gly-OEt	210	14	Leu-Leu	11	5.5
Ala-Thr	1.6	6			
Ala-Thr-Ala	190	3.2			
Ala-Thr-Ala-OMe	170	2.7			
Leu-Gly-Leu	90	7			

* Expressed relative to Ala-Gly-Gly as 100.

Discussion

Yeast aminopeptidase I, the purification and characteristics of which are described, is responsible for about 90% of the yeast cell's aminopeptidase activity. No evidence could be found supporting the existence of another N-terminal peptidase of similar importance in yeast. Moreover, a preparation we obtained as described by Grassmann et al. [4] proved to be identical to the enzyme resulting from both our and Johnson's [7] purification method. In the present paper it is shown that the enzyme's activity is very sensitive to the presence of activators. Thus, divergent results on the substrate specificities of earlier preparations [5,7,8] most probably are due to some differences in the purification or assay procedures applied, rather than to real differences between the enzymes under study.

Aminopeptidase I (so called by us because it was the first yeast peptidase characterized in some detail) resembles the well-known leucine aminopeptidases not only in its substrate specificity but likewise with respect to some other features common to many enzymes of this group [31]. So the molecular weights of the leucine aminopeptidases, which frequently are glycoproteins [33,34], are on the average much higher than those of typical proteinases [31, 34–36]. The yeast enzyme with a M_r of 600 000 is, so far as we know, the largest peptidase molecule found as yet.

Another property of most aminopeptidases is their dependence on divalent metal ions. In some cases evidence could be presented indicating the existence of functionally different metal binding sites on the same enzyme [37,38]. Although futher experiments are necessary to establish this point, it seems that yeast aminopeptidase I behaves in the same way, containing a few strongly bound metal ions involved in catalysis, but being able to undergo an additional activation by the binding of further Zn^{2+}. A mainly structural role of the activating metal, similar to the "tightening" effect of MN^{2+} or Mg^{2+} on *E. coli* glutamine synthetase [39], is suggested by our results.

Activation by halide ions is also a usual phenomenon with peptidases [40–42]. Sometimes a nearly absolute requirement for these ions is observed. The mechanism underlying the halide activation of yeast aminopeptidase, that is, a positive allosteric effect abolishing the enzyme's cooperativity with respect to its substrates, has been previously demonstrated to hold for dipeptidyl aminopeptidase I (cathepsin C) [42]. In our case the conformational adjustment accompanying the halide effect seems to be less extensive than the conformative response upon binding of Zn^{2+}.

Finally, it is interesting to note that the highest relative activation by both Zn^{2+} and Cl^- is observed below pH 6, that is, in the range of pH which prevails within the yeast vacuole where a large fraction of the enzyme is located in vivo [43]. So at pH 5.0 a more than 350-fold rate enhancement is brought about by the combined effects of both activators.

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