Yeast Aminopeptidase II: Rapid Purification Using Affinity Chromatography of the Periplasmic Enzyme

Joseph Knüver and Klaus-Heinrich Röhm

Institut für Physiologische Chemie der Universität, Lahnberge, D-3550 Marburg (Lahn), Bundesrepublik Deutschland

Z. Naturforsch. **37 c**, 845–848 (1982);
received March 25, 1982

Yeast Aminopeptidase II, Affinity Chromatography, Bestatin

Periplasmic aminopeptidase II of yeast was isolated from protoplast supernatants by ammonium sulfate precipitation, DEAE-Sephacel chromatography and affinity chromatography on immobilized bestatin. This isolation method is more rapid than conventional procedures and, in addition, avoids contact of the enzyme with yeast proteinases. Thus the preparation obtained is likely to represent the native periplasmic from of aminopeptidase II.

Introduction

Aminopeptidase II is the main aminopeptidase of growing *Saccharomyces* yeasts [1]. Most of the enzyme is found in the periplasmic compartment, i.e. outside the plasma membrane [2]. This particular location suggests that it is involved in nitrogen assimilation by degrading external peptides. A similar aminopeptidase was recently found in *Schizosaccharomyces pombe* [3].

Attempts to purify aminopeptidase II from cell homogenates met considerable difficulties due to rapid inactivation of enriched preparations. Nevertheless, two enzyme forms were eventually obtained in homogeneous state [4, 5]. They were indistinguishable with respect to catalytic behaviour but differed in molecular weight (100 000 and 140 000). The analogy with other periplasmic hydrolases suggested that the isoenzymes of aminopeptidase II might be its periplasmic form and a cytosolic precursor. On the other hand, we could not exclude that the observed heterogeneity was the result of limited enzymic degradation, taking place during the lengthy purification procedure. Thus, in order to obtain periplasmic aminopeptidase II in its native state, we followed a different strategy. Instead of homogenisation, we now released the enzyme from intact cells by enzymic digestion of the cell wall with snail gut enzymes. Second, to speed up the isolation procedure, we introduced affinity chromatography on immobilized bestatin [6]. In this way highly purified periplasmic aminopeptidase II was obtained in only three steps.

Reprint requests to Dr. K.-H. Röhm.

Abbreviations: Ahp, 3-amino-2-hydroxy-4-phenylbutanoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

0341-0382/82/0900-0845 $ 01.30/0

Materials and Methods

A strain, isolated from commercial brewer's yeast in our laboratory, was used for all experiments. The cells were grown in a 10-l fermentor using 'complex' medium (2% glucose, 1% peptone, 1% yeast extract). Protoplasts were obtained from log-phase cultures by treatment with 'glusulase' (β-glucuronidase/arylsulfatase from *Helix pomatia*, Serva, Heidelberg, FRG). With 0.5 ml 'glusulase'/g yeast (wet weight) about 80% of the cells were converted to protoplasts within 30 min. The resulting protoplast suspension was layered on the 4-fold volume of 0.6 M sucrose and 2% Ficoll in 20 mM HEPES/Tris buffer, pH 7.0. Protoplasts were sedimented by centrifugation (10 min at $3000 \times g$). The top layer of the tubes, containing the released enzyme, was cleared by high-speed centrifugation and used as starting material for aminopeptidase II purification. The protoplasts were washed by two further centrifugations through sucrose/Ficoll/HEPES and then lysed by sonication.

Aminopeptidase II activities were assayed as described elsewehere [1]. On polyacrylamide gels the enzyme was detected by activity staining: The gels were incubated in buffered 0.5 mM lysine-β-naphthylamide (20 min at 40 °C) and after that transferred to a staining solution of 0.1% Fast Garnet GBC (Serva) and 1.5% Brij 35 in 0.33 M acetate buffer, pH 4.2. Bright red bands of azo dye appeared at the location of aminopeptidase II.

An affinity adsorbent was obtained by coupling [2RS, 3S]-Ahp-L-Leu, i.e. a racemic mixture of two out of 8 stereoisomers of bestatin, to AH-Sepharose 4B (Pharmacia, Uppsala, Sweden): First, [2RS, 3S]-Ahp was synthesized from L-Phe as described by Suda *et al.* [7]. It was converted to the Boc-derivative by treatment with di-t-butylpyrocarbonate [8], and then coupled to L-Leu-OBzl · Tos by the carbodiimide method [9]. The C-terminal ester group of Boc-Ahp-L-Leu-OBzl was removed by catalytic

hydrogenation to give Boc-[2RS, 3S]-Ahp-Leu in 20% yield (with respect to Ahp). Hydrated AH-Sepharose (4 ml) and Boc-protected ligand (200 mg) were suspended in 10 ml dioxane/water 1:4. EDC · HCl (300 mg) was added and pH adjusted to 5.4. The reaction mixture was incubated overnight with agitation and washed with dioxane/water and water. The resulting material was shaken with 20 mg/ml EDC · HCl in 0.1 M acetate buffer, pH 4.6 for another day to block the remaining free amino groups. Finally, the Boc-protective groups were removed by treatment with 0.5 N HCl in methanol (1 h at 40 °C). The material contained about 1 μmol ligand/ml gel as determined by amino acid analysis of a hydrolysate (in 6 N HCl, 24 h at 110 °C).

Aminopeptidase II was purified from the protoplast supernatant of 25 g yeast (wet weight) as follows:

a) The supernatant was dialyzed against saturated ammonium sulfate solution overnight. The precipitate formed was washed with 90% $(NH_4)_2SO_4$ and then dissolved in 10 mM Tris/succinate, pH 7.3, followed by exhaustive dialysis against the same buffer.

b) A 2 × 30 cm column of DEAE-Sephacel (Pharmacia) was equilibrated with 10 mM Tris/succinate, pH 7.3 as usual. The protein solution was applied at a rate of 30 ml/h, followed by buffer until no further protein emerged. Aminopeptidase II was eluted by 250 ml of a linear NaCl gradient (0 – 200 mM). The active fractions were pooled, concentrated to 5 ml by ultrafiltration, diluted 30-fold with 10 mM 3,3′-dimethyl-glutarate/NaOH, pH 7.0 and again concentrated to a few ml.

c) Ahp-Leu-AH-Sepharose was packed in a small column (1 × 2 cm) and equilibrated with dimethylglutarate/NaOH buffer. 0.5 – 1 ml of enzyme solution was applied (flow rate: 5 ml/h). After unbound protein was washed off by dimethylglutarate buffer, aminopeptidase II was eluted with 10 mM Leu-Gly-Gly (Serva) in buffer.

Results and Discussion

Subcellular distribution of aminopeptidase II in brewer's yeast

Previous experiments with *S. cerevisiae* NCYC 366 [2] showed that, with this strain, about half of the enzyme is located exocellularly. The strain of brewer's yeast used here gave different results: Lysates of protoplasts, carefully washed free of cell wall remnants, contained very little aminopeptidase II when lysed (<10% of total). This discrepancy is yet unexplained. Possibly, the distribution of the enzyme varies from yeast to yeast, or depends on nutrional state and age of the cultures.

Purification

The method outlined above yielded highly purified aminopeptidase II in only three days. The resulting enzyme had the same specific activity as the best preparations obtained by conventional procedures before (Table I). Overall purification was 400-fold with yields up to 40%. In contrast, isolation from yeast homogenates took 3–4 weeks, and not more than a few percent of the activity were eventually recovered.

Chromatography on DEAE-Sephacel performs a dual role. It removes the bulk of protein and separates aminopeptidase II and an aminopeptidase present in 'glusulase'. The latter enzyme, being much

Table I. Purification of periplasmic aminopeptidase II. Substrate: 0.5 mM Lys-*p*-nitroanilide, 40 °C, pH 7.0.

Preparation	Volume [ml]	Protein [mg]	Activity [mU]	Spec. Act. [mU/mg]	Recovery [%]	Purification factor
Protoplast supernatant	56	615	800[a]	1.3	100	1
DEAE-Sephacel eluate	4	26	520	20	65	15
Affinity chromatography[b]	5	0.6	305	510	38	390

[a] Corrected for the aminopeptidase activity of 'glusulase', see text.
[b] Sum of four runs with 1-ml samples

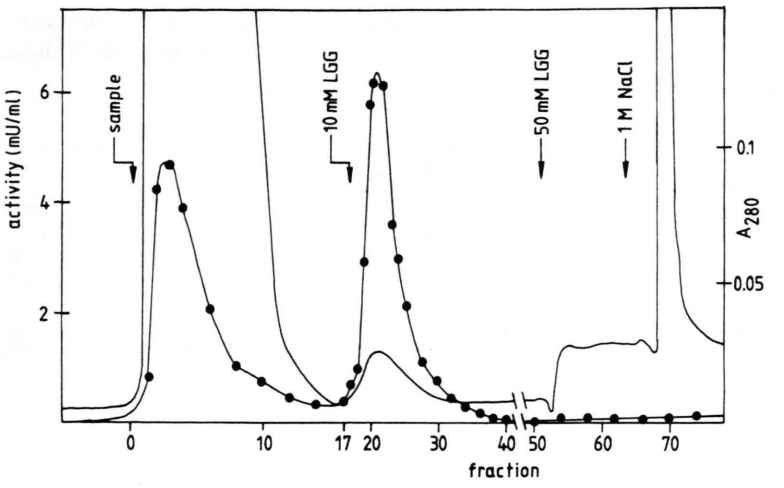

Fig. 1. Affinity chromatography of periplasmic aminopeptidase II on (2RS, 3S)-Ahp-Leu-aminohexylsepharose. An 1-ml sample of material from DEAE-Sephacel chromatography was applied to a 0.2×3 cm micro-column. The bulk of protein and, due to overloading, some activity appears in the breakthrough peak. The bound enzyme was specifically eluted by 10 mM Leu-Gly-Gly. Starting from tube 17, fraction size was reduced from 0.6 ml to 0.3 ml. Protein was monitored by the absorption at 280 nm (——); aminopeptidase activity (●) was measured with lysine-*p*-nitroanilide as substrate.

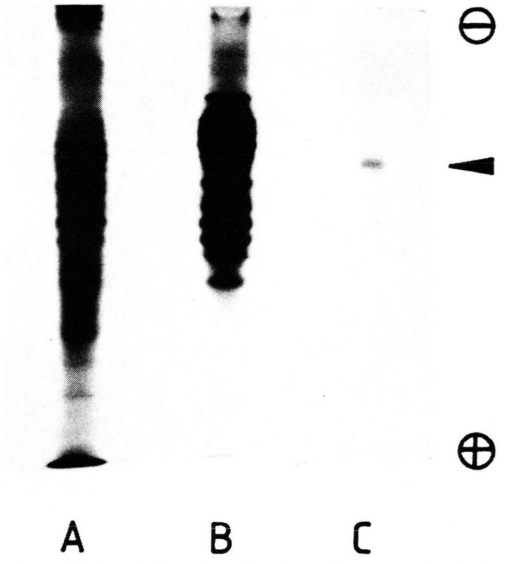

Fig. 2. Gel electrophoresis of aminopeptidase II preparations. Disk electrophoresis was performed in a 7.5% polyacrylamide gel at pH 8.9, using standard procedures. The gel was stained for protein with Coomassie Blue G 250. A) protoplast supernatant, B) DEAE-Sephacel eluate, C) enzyme purified by affinity chromatography. Activity staining showed a single band of aminopeptidase II with a mobility as indicated by the arrow.

more acidic than aminopeptidase II, requires 200 mM NaCl to be eluted from DEAE-Sephacel, whereas the yeast aminopeptidase appears between 30 and 50 mM. Under the conditions chosen for protoplast formation, snail aminopeptidase was in about 3-fold excess over aminopeptidase II. This ratio was taken into account when calculating purification factors and yields of Table I.

The efficiency of the affinity chromatography is clearly illustrated by Figs. 1 and 2. Aminopeptidase II was selectively eluted by a peptide substrate, Leu-Gly-Gly; some inactive material appeared with 1 M NaCl. This confirms that interaction of the enzyme with Ahp-Leu-AH-Sepharose is, in fact, based on affinity effects; electrostatic binding was of little importance. All of the aminopeptidase II activity eluted by Leu-Gly-Gly migrated in the main protein band when analyzed by gel electrophoresis. (Fig. 2, arrow), Some additional faint bands were usually observed, none of them active. They could be largely removed by repeating the affinity step with dialyzed Leu-Gly-Gly eluate.

Properties

Periplasmic aminopeptidase II as produced by the above method, was indistinguishable from the smaller one of the isoenzymes described earlier [5]. Gel filtration through Sephacryl S-300 gave a single symmetric peak with a molecular weight close to 100 000. The larger form (M_r 140 000) was not detected. A comparison of substrate specifities and kinetic parameters showed no significant differences between enzymes purified by affinity chromatography or by conventional means. On the other hand, the stability of aminopeptidase II preparations is greatly improved when the enzyme is isolated as detailed above. Even dilute solutions could be stored in the cold for weeks without appreciable loss of activity. This suggests that, in fact, proteolytic degradation is responsible for the poor yields of

preparations starting from cell homogenates. The main proteinase activities of yeast, together with other hydrolases, are sequestered in the cell vacuole [10]. Aminopeptidase II, when released by enzymic cell wall degradation, never gets in touch with these vacuolar enzymes.

[1] J. Frey and K.-H. Röhm, Biochim. Biophys. Acta **527,** 31–41 (1978)
[2] J. Frey and K.-H. Röhm, Eur. J. Biochem. **97,** 169–173 (1979).
[3] D. Atmanspacher and K.-H. Röhm, Hoppe-Seyler's Z. Physiol. Chem. **362,** 459–463 (1981).
[4] J. Knüver, Diplomarbeit, University of Marburg, Chemistry Department 1979.
[5] J. Knüver and K.-H. Röhm, Hoppe-Seyler's Z. Physiol. Chem. **361**, 285–286 (1980).
[6] H. Umezawa, T. Aoyagi, H. Suda, M. Hamada, and T. Takeuchi, J. Antibiot. **29,** 97–99 (1976).
[7] H. Suda, T. Takita, T. Aoyagi, and H. Umezawa, J. Antibiot. **29,** 600–601 (1976).
[8] L. Moroder, A. Hallett, E. Wünsch, O. Keller, and G. Wersin, Hoppe-Seyler's Z. Physiol. Chem. **357,** 1651–1653 (1976).
[9] R. Nishizawa, T. Saino, T. Takita, H. Suda, T. Aoyagi, and H. Umezawa, J. Med. Chem. **20,** 510–515 (1977).
[10] J. F. Lenney, P. Matile, A. Wiemken, M. Schellenberg, and J. Meyer, Biochem. Biophys. Res. Commun. **60**, 1378–1383 (1974).

Verantwortlich für den Inhalt: A. KLEMM
Satz und Druck: Konrad Triltsch, Würzburg