Purification and Characterization of a Methionine Aminopeptidase from Saccharomyces cerevisiae*

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Methionine aminopeptidase (MAP), which catalyzes the removal of NH₂-terminal methionine from proteins, was isolated from Saccharomyces cerevisiae. The enzyme was purified 472-fold to apparent homogeneity. The M₆ of the native enzyme was estimated to be 36,000 ± 5,000 by gel filtration chromatography, and the M₆ of the denatured protein was estimated to be 34,000 ± 2,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme has a pH optimum near 7.0, and its pI is 7.8 as determined by chromatofocusing on Mono P. The enzyme was inactivated by metalloprotease inhibitors (EDTA, o-phenanthroline and nitrilotriacetic acid), sulfhydryl-modifying reagents (HgCl₂ and p-hydroxymercuribenzoic acid), and Zn⁺⁺. Yeast MAP failed to cleave methionine p-nitroanilide. Among 11 Xaa-Ala-Ser analogues (Xaa = Ala, Asp, Gln, Glu, Ile, Leu, Lys, Met, Phe, Pro, and Ser), MAP cleaved only Met-Ala-Ser. MAP also cleaved methionine from other tripeptides whose penultimate amino acid residue is relatively small and/or uncharged (e.g. Pro, Gly, Val, Thr, or Ser) but not when bulky and/or charged (Arg, His, Leu, Met, or Tyr). Yeast MAP displayed similar substrate specificities compared with those of Escherichia coli (Ben-basset, A., Rauer, K., Chang, S. Y., Myambo, K., Boosman, A., and Chang, S. (1987) J. Bacteriol. 169, 751-757) and Salmonella typhimurium MAP (Miller, C., Strauch, K. L., Kukral, A. M., Miller, J. L., Wingfield, P. T., Mazzei, G. J., Werlen, R. C., Garber, P., and Movva, N. R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2718-2722). In general, the in vitro specificity of yeast MAP is consistent with the specificity observed in previous in vivo studies in yeast (reviewed in Arfin, S. M., and Bradshaw, R. A. (1988) Biochemistry 27, 7970-7984).

During translation, newly synthesized proteins contain either an NH₂-terminal formylmethionine (prokaryotic proteins) or a methionine (eukaryotic proteins) residue (1-11). In the case of prokaryotic proteins, the formyl group is usually removed by a deformylase, leaving a methionine residue bearing a free α-NH₂ group (7-11). NH₂-terminal methionine is subsequently cleaved from many, but not all, prokaryotic and eukaryotic proteins by a methionine aminopeptidase (MAP)1 (1-5).

Analyses of protein sequence data from disparate proteins, as well as data from model protein systems indicate that MAPS from both prokaryotes and eukaryotes share similar substrate specificities. MAPS are capable of removing the NH₂-terminal methionine when the penultimate amino acid residue is relatively small and/or uncharged (e.g. Gly, Ala, Pro, Ser, Thr, Val, and Cys) but not when the residue is relatively bulky and/or charged (1-5, 12-14; for a review, see Ref. 15). Although a functional role for methionine remains unclear, Varshavsky and co-workers (16, 17) have suggested that NH₂-terminal methionine may play a role in stabilizing certain cytosolic eukaryotic proteins whose penultimate residues are bulky and/or charged.

The gene encoding MAP from both Escherichia coli and Salmonella typhimurium were cloned and sequenced (18, 19). However, the purification of MAP from a eukaryotic organism has not been previously achieved due to the instability of MAP, as well as contamination with nonspecific aminopeptidases (15, 20).

In this paper we report the first complete purification, as well as the partial characterization of a MAP from a eukaryotic organism, Saccharomyces cerevisiae. This purified enzyme allowed us to clone, sequence, and study the essentiality of the MAP1 gene.² The availability of this purified enzyme will also allow us to generate polyclonal antibodies to be used in studies aimed at determining the interaction of MAP with ribosomes and other acylating enzymes.

EXPERIMENTAL PROCEDURES³

RESULTS AND DISCUSSION

Purification and Molecular Properties—A MAP was purified to apparent homogeneity from S. cerevisiae, as described under "Experimental Procedures." The extreme instability of MAP in the crude extract from fresh yeast cells complicated

1 The abbreviations used are: MAP, methionine aminopeptidase; buffer H, a buffer containing 10 mM Hepes, pH 7.35, 1.5 mM MgCl₂, 10% glycerol, and 0.02% NaN₃; Boc, t-butoxycarbonyl; CP-A, carboxypeptidase A; DTT, dithiothreitol; Fmoc, 9-fluorenylmethoxycarbonyl; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; LAP, leucine aminopeptidase; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; FMSF, phenylmethanesulfonyl fluoride; pNA, p-nitroanilide; pMB, p-hydroxymercuribenzoic acid; SDS, sodium dodecyl sulfate; TLCK, N-tosyl-L-lysine chloromethyl ketone.

2 V. H. Chang, 17; Twicher, and J. A. Smith, unpublished observations.

³ Portions of this paper (including "Experimental Procedures" and Figs. 1-4, 6-8, and Tables 2-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Purification and Characterization of a Yeast MAP

our initial purification (data not shown). However, the enzyme could be readily purified from yeast cells stored at −20 °C for at least 4 months. It is unclear why prolonged storage at −20 °C enhanced the apparent stability of MAP, but inactivation of proteases capable of degrading MAP or processing of native MAP to a more stable form are possible explanations. As shown in Table 1, a multiple-step purification from 200 g (wet weight) of yeast cells resulted in a 472-fold purification. The overall low fold purification can be accounted for by the presence of several contaminating leucine aminopeptidases (LAPs) in the crude extract (26, 27), which each readily degraded Met-Ala-Ser, the substrate used for assaying MAP activity. These LAPs were separated from MAP by the CM-Sepharose chromatography step.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals a single Coomassie Blue-stained band with an \( M_r = 34,000 \pm 2,000 \) (Fig. 5, lane 2). Gel filtration chromatography shows that the \( M_r \) of the native MAP is 36,000 ± 5,000 (Fig. 6). These data indicate that MAP is monomeric and that its \( M_r \) is similar to prokaryotic MAPs from E. coli and S. typhimurium (32,000 and 35,000, respectively) (18, 28). However, the calculated molecular weights for both prokaryotic MAPs, derived from their deduced amino acid sequences, are considerably smaller (29,333 and 29,292, respectively) (18, 28). Whether a similar discrepancy for yeast MAP will be observed and what is the nature of the presumed post-translational modification remain to be established.

Chromatofocusing on Mono P revealed a broad peak at apparent \( pI = 7.8 \) (Fig. 7), which is different from the apparent \( pI \) (5.4) determined for the S. typhimurium MAP (28).

The \( pI \) dependence of yeast MAP was measured by assay- ing at various \( pH \) values from 5 to 8.5 (Fig. 8A), and maximal enzyme activity was observed at \( pH 7 \). Assays for determining the temperature optimum for yeast MAP were performed from 4 to 55 °C (Fig. 8B), and the yeast MAP displays maximum activity at temperatures from 30 to 45 °C.

The amino acid composition of the enzyme shown in Table 2 is characteristic of a globular protein, and differs from the amino acid compositions of the E. coli and S. typhimurium MAPs (18, 28).

**Effect of Protease Inhibitors and Divalent Cations on Enzyme Activity**—As shown in Table 3, EDTA, 2-phenanthroline and NTA (i.e. metal ion chelating reagents) inactivated MAP, indicating that MAP is a metalloprotease. In addition, yeast MAP was not inhibited by pepstatin, chymostatin, antipain, leupeptin, Trasylol, TLCK, or bestatin (i.e. an inhibitor of LAPS) (data not shown).

MAP was readily inactivated by sulfhydryl-modifying reagents, 
\( \text{HgCl}_2 \) and pHMB (Table 3). Hence, a sulfhydryl group may be present in catalytic residues or coordination sites for active-site metal ion binding.

MAP can also be completely inactivated by 10 mM ZnCl\(_2\) (Table 3). Recently, it was shown that Zn\(^{2+}\) ions can inhibit another metalloprotease, carboxypeptidase A, after the formation of ZnOH\(^+\), which binds to the carboxylate involved in metal ion binding and forms a stable hydrogen bond between the inactivating Zn\(^{2+}\) ion and the catalytic metal ion (30). Whether Zn\(^{2+}\) inhibits MAP by a similar mechanism remains to be established.

To further examine the metal ion requirement of MAP, the endogenous metal ion(s) was removed by dialysis against 1 mM EDTA, which decreased the enzyme activity of MAP (Table 4). After incubation of inactivated MAP with 3 mM CoCl\(_2\), MAPs enzyme activity increased to 37%, although activation was not observed with MgCl\(_2\), MnCl\(_2\), CuCl\(_2\), FeCl\(_2\), and ZnCl\(_2\) (Table 4). These data indicate that Cu\(^{2+}\) likely plays an important role in the catalysis and stability of the eukaryotic MAP, as it does for the MAP from E. coli or S. typhimurium (18, 28).

**Substrate Specificity of MAP**—Nine amino acid \( p \)-nitroanilides and 28 peptide substrates (differing in sequence and length) were used to measure the activity of yeast MAP in vitro (Table 5).

As shown in Table 5, yeast MAP showed no detectable activity toward any of the representative amino acid \( p \)-nitroanilides (pNA) (Ala, Arg, Glu, Gly, Leu, Lys, Met, Pro, or Val), indicating that MAP is different from all other yeast aminopeptidases (for review, see Ref. 27).

Among 11 representative tripeptide substrates, Xaa-Ala-Ser (Xaa = Ala, Asp, Gln, Glu, Ile, Leu, Lys, Met, Phe, Pro, and Ser), MAP cleaved only the Met-Ala peptide bond in Met-Ala-Ser, indicating that MAP was capable of cleaving only \( NH_2 \)-terminal methionine. In addition, MAP cleaved NH\(_2\)-terminal methionine from other tripeptides whose penultimate amino acid residue is relatively small and/or uncharged (e.g. Ala, Pro, Gly, Val, Thr, or Ser) but not when the residue is relatively bulky and/or charged (Arg, His, Ile, Leu, Met, or Tyr) (Table 5). These results are consistent with the substrate specificity of the MAPs from E. coli and S. typhimurium (18, 19) and are, in general, consistent with the results obtained by other investigators, who have analyzed the \( NH_2 \)-terminal sequence data of various mutant forms of recombinant proteins (12–15).

Yeast MAP cleaved Met-Ala and Met-Ser, albeit inefficiently, although E. coli MAP did not cleave these substrates (18). Furthermore, Huang et al. (12) observed that methionine followed by Thr, Ser, or Ala was efficiently removed in the recombinant plant thaumatin variants expressed in yeast, although in vivo yeast MAP displayed about 20 times higher activity toward Met-Ala-Ser than Met-Thr-Ser or Met-Ser-Gly. Whether this disagreement is simply due to differences in the in vivo versus in vitro assays or whether there is another MAP that is responsible for the specific removal of methionine followed by Thr or Ser is presently unclear.

As shown in Table 5, both the length of the peptide substrate (e.g. Met-Ala (6%), Met-Ala-Ile (30%), Met-Ala-ile-Pro (8%), and Met-Ala-Ile-Pro-Glu (64%)), as well as type of residues which resides at more distal positions can affect the activity of MAP (Met-Ala-Ser (100%) and Met-Ala-Ile (30%);
Purification and Characterization of a Yeast MAP

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified yeast MAP. The electrophoresis was performed according to the method of Laemmli (23) using a 10% gel. The gel was stained with Coomassie Blue. Lane 1, molecular weight standards (from the top): myosin (205,000), E. coli β-galactosidase (116,000), rabbit muscle phosphorylase (97,000), bovine serum albumin (66,000), and egg albumin (45,000); lane 2, purified MAP (3 μg).

The gel was stained with Coomassie Blue. Lane I, molecular weight standards (from the top): myosin (205,000), E. coli β-galactosidase (116,000), rabbit muscle phosphorylase (97,000), bovine serum albumin (66,000), and egg albumin (45,000); lane 2, purified MAP (3 μg).

Table 5

<table>
<thead>
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<th>Substrate</th>
<th>Activity</th>
<th>Substrate</th>
<th>Activity</th>
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</thead>
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<tr>
<td>Met-Ala-Ser</td>
<td>100 ± 6</td>
<td>Xaa-Ala-Ser&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Xaa-pNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>Met-Ala</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Met-Pro-Gly</td>
<td>36 ± 4</td>
<td>Met-Arg-Phe</td>
<td>0</td>
</tr>
<tr>
<td>Met-Gly-Gly</td>
<td>26 ± 3</td>
<td>Met-His-Gly</td>
<td>0</td>
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<tr>
<td>Met-Thr-Ser</td>
<td>6 ± 2</td>
<td>Met-Leu-Gly</td>
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<td>Met-Val-Ser</td>
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<td>Met-Ser-Gly</td>
<td>3 ± 2</td>
<td>Met-Tyr-Lys</td>
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<tr>
<td>Met-Ala-Ile</td>
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<td>Met-Ala-Ile-Pro-Glu</td>
<td>64 ± 4</td>
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<tr>
<td>Met-Ala-Ile-Glu</td>
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<td>Met-Ala-Ile-Pro-Ser</td>
<td>36 ± 4</td>
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<tr>
<td>Met-Ala-Ile-Pro</td>
<td>8 ± 2</td>
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</table>

<sup>a</sup> Met-Ala-Ser: reference peptide.
<sup>b</sup> Xaa: Ala, Asp, Gin, Glu, Ile, Leu, Lys, Phe, Pro, Ser.
<sup>c</sup> Xaa: Ala, Arg, Glu, Gly, Leu, Lys, Met, Pro, Val.

Met-Ala-Ile-Glu (12%) and Met-Ala-Ile-Pro (8%); and Met-Ala-Ile-Pro-Glu (64%) and Met-Ala-Ile-Pro-Ser (36%).

The MAP from E. coli was similarly affected by distal residues (Met-Gly-Met-Met > Met-Gly-Met > Met-Gly-Gly) (18).

In summary, although the prokaryotic and eukaryotic MAPs have a requirement for Co<sup>2+</sup> and similar substrate specificities, they differ in their pl values (5.4 versus 7.8, respectively) and amino acid compositions. In order to eluci-
Experimental Procedures

Chromatography on Mono P
Partially purified enzyme, purified through the Sepharose 2-200 chromatography, was diluted against 50 mM Tris-acetate buffer (pH 7.3) containing 50% glycerol. The sample (10 ml) was applied to Mono P (5.2 × 1.5 cm) equilibrated with the above buffer at a rate of 0.5 ml/min at 4°C. Elution was monitored by A280, and 1 ml fractions were collected for measurement of pH and enzyme activity.

Peptide Synthesis
Leu-Ala-Ser, Pro-Ala-Ser, Phc-Ala-Ser, and Meta-Ala-Tyr were synthesized using Fmoc solid-phase synthesis on a Millipore MilliSieve 3003 peptide synthesizer, as previously described [24]. Leu-Ala, Ser-Ala, Ser-Tyr, Gly-Ala, Ala-Ala, Tyr-Ala, Pro-Ala, and Tyr-Pro were prepared as previously described [25].

Methods

UV measurements were obtained using a Saffit-Packard 1603 and spectrophotometer. Protein assays were performed by the method of Bradford [26]. Using bovine albumin as the standard.

The enzyme assay is a modification of the method of Carter and Miller [22]. Using bovine albumin (the culture filtrate was not adjusted to pH 7.0). The solutions were kept on ice throughout the procedure. The results were expressed as micromoles of peptide synthesized per milligram of protein.

Cell harvest
Concentrated culture (0.8 liter) was thawed in a 30°C water bath, and the culture filtrate was autoclaved (24°C, 15 lb per sq in) for 60 min. The cells were then centrifuged (25,000 g, 1 h, 0°C) and the cell suspension was added to 4.5 liters of 50°C buffer (0.25 M Tris-HCl, pH 7.5, 1 M NaCl, and 0.02% NaN3) and the time points were adjusted to 30 min. Then the cells were harvested with a sterile syringe, resuspended in 1 liter of 0.1 M Tris-HCl, pH 7.5, and the enzyme was assayed immediately.

SDS-PAGE and Western blotting

A sample of purified WAP was loaded on a 10% PAGE gel (10 μg) and electrophoresed under reducing conditions as described by Laemmli [33], and stained with Coomassie Blue R-250. The gel was then subjected to Western blotting using the procedure of Towbin et al. [34]. The following proteins were used as standards including 50,000, 35,000, 25,000, 20,000, and 14,000 Daltons.

Molecular size determination

The M, of WAP was determined by comparison with molecular weight standards of gel filtration on a Sepharose 6B column (1.0 × 10 cm) in 0.1 M Tris-acetate buffer containing 0.1 M NaCl (pH 7.3).

Supplemental Material to

Purification and Characterization of a Methionine Amidopeptidase

From Bacillus subtilis

by Tae-Soo Chang, Ulrich Velichet and John A. Smith

Chromatography on Mono P

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Purification and Characterization of a Yeast MAP

Figure 2. Hydroxyapatite chromatography of yeast MAP. The MAP pooled from CM-Sepharose (1-68, 10.0 to 0.2 M KC1) was concentrated, dialyzed, and applied to a hydroxyapatite column, eluted with a linear gradient of 0.1-1.0 M KC1 to 0.5 M 1.25 M potassium phosphate buffer, pH 7.5; 100 mM KCl, 100 μl mlol, 0.1% NaN3, at 5 ml/hr with a flow rate of 0.2 ml/hr at 4°C. Elution was monitored by A 280 and AU 400. Fractions containing MAP activity were pooled as indicated by horizontal bar.

Figure 3. Sepharose S-200 chromatography of yeast MAP. The MAP pooled from hydroxyapatite column was concentrated and applied to a Sepharose S-200 column. Eluted with buffer K containing 1.2 M KC1 and 3.5 mg/ml Cel1 at 1.1 ml/hr at 4°C. Elution was monitored by A 280. Fractions containing MAP activity were pooled as indicated by horizontal bar.

Figure 4. Red-Sepharose CL-6B chromatography of yeast MAP. The MAP pooled from Sepharose S-200 column was applied to a Red-Sepharose CL-6B column, eluted with a linear gradient of 0.1-1.0 M KC1 to 0.5 M 1.25 M potassium phosphate buffer, pH 7.5; 100 mM KCl, 100 μl mlol, 0.1% NaN3, at 5 ml/hr with a flow rate of 0.2 ml/hr at 4°C. Elution was monitored by A 280 and AU 400. Fractions containing MAP activity were pooled as indicated by horizontal bar.

Figure 5. Determination of the molecular weight of native yeast MAP by gel filtration. Partially purified enzyme pooled from hydroxyapatite column was applied to a Red-Sepharose CL-6B column, eluted with buffer K containing 1.2 M KC1 and 3.5 mg/ml Cel1 at 1.1 ml/hr at 4°C. Elution was monitored by A 280. Fractions containing MAP activity were pooled as indicated by horizontal bar.

Figure 6. Anthropoassay of yeast MAP. The MAP pooled from Sepharose S-200 column was applied to a Red-Sepharose CL-6B column, eluted with a linear gradient of 0.1-1.0 M KC1 to 0.5 M 1.25 M potassium phosphate buffer, pH 7.5; 100 mM KCl, 100 μl mlol, 0.1% NaN3, at 5 ml/hr with a flow rate of 0.2 ml/hr at 4°C. Elution was monitored by A 280 and AU 400. Fractions containing MAP activity were pooled as indicated by horizontal bar.

Figure 7. Chromatofocusing of yeast MAP on a Mono P column. The partially purified enzyme from Sepharose S-200 was applied to a Mono P column (100-5.5), equilibrated with 50 mM Tris buffer, pH 8.5, and eluted with a gradient of 500 mEq ammonium acetate buffer, pH 8.5, at the flow rate of 0.5 ml/min at 4°C. Elution was monitored by A 280. Fractions containing MAP activity were pooled as indicated by horizontal bar.

Figure 8. Effect of pH and temperature on yeast MAP. (A) The relative enzyme activity of yeast MAP for histidase was determined in Tris-maleate buffers (pH 5-8.5), as described in "Experimental Procedures". (B) The relative enzyme activity of yeast MAP for Metidase was determined at different temperatures, as described in "Experimental Procedures".

Table 1

<table>
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<tr>
<th>Anthranilic Acid Composition of MAP from S. cerevisiae</th>
<th>Measured Residuea</th>
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<tbody>
<tr>
<td>Alanine</td>
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<td>Threonine</td>
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<td>Glutamic acid</td>
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<tr>
<td>Glutamine</td>
<td>35.3</td>
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<tr>
<td>Aspartic acid</td>
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<td>Asparagine</td>
<td>31.6</td>
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<tr>
<td>Histidine</td>
<td>21.7</td>
</tr>
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</table>

a The amino acid compositions of peptides were determined from three different enzyme preparations using an Amino X HPLC Analyzer after 24 h in hydrolysates at 110°C in 6 N HCl containing 0.1% phenol (G1). Amino Acid Identification

b The number of MAP was calculated on the basis of a M, 250,000. The determination was made for the amount of MAP and the amount, of the 24 h treatment. The activity of MAP and glycosidase were not determined.

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[Image 0x-2 to 591x806]
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### Table 3

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<td>Tris-HCl</td>
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### Table 4

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<td>Tris-HCl</td>
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* Yeast methionine synthase was purified according to the procedure of Nair et al. (1990). The enzyme activity was determined under standard assay conditions, using homocitrulline as the substrate.
Purification and characterization of a methionine aminopeptidase from *Saccharomyces cerevisiae*.
Y H Chang, U Teichert and J A Smith


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