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Peptidase Activities in *Saccharomyces cerevisiae*

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At least four distinct aminopeptidase activities and a single dipeptidase activity were found in cell extracts of a leucine–lysine auxotroph of *Saccharomyces cerevisiae*. The assay for peptidase activity involved polyacrylamide gel electrophoresis followed by an enzyme-coupled activity staining procedure. The aminopeptidases had largely overlapping specificities but could be distinguished from one another by their electrophoretic mobilities and activities toward different peptide substrates. Substrates tested included both free and blocked di- and tripeptides and amino acid derivatives.

It has been demonstrated by several workers that various microorganisms, including yeasts, contain aminopeptidase, carboxypeptidase, and dipeptidase activities (11, 15, 20, 23, 25, 26, 28). Peptidases are capable of providing amino acids for cell growth by hydrolyzing peptides. There is also evidence that peptidases are constitutive enzymes and that they are present in concentrations higher than those required for cell growth on peptides (27, 29). Thus, it has been speculated that peptidases have a number of physiological roles. These include degradation of proteins with incorrect primary sequences, protection of organisms from the toxic effects of certain low-molecular-weight peptides, and the final cleavage of N-terminal methionyl residues from nascent protein chains in bacteria (30).

A fairly detailed electrophoretic analysis of aminopeptidases in *Saccharomyces cerevisiae* used leucyl-2-naphthylamide as the substrate (16). The use of this substrate, as well as other amino acid derivatives, may lead to erroneous conclusions because these substrates are susceptible to hydrolysis by enzymes which are not true peptidases (22). More recently, Frey and Rohm (6) demonstrated the existence of three aminopeptidases and a single dipeptidase in partially fractionated cell extracts of *S. cerevisiae*. Several yeast peptidases have also been purified and characterized to varying degrees (3, 5, 9, 15, 17, 24). However, we still do not know the total number and types of peptidases present in *S. cerevisiae*. In this communication we report on the number of independent peptidase activities present in a leucine–lysine auxotroph of *S. cerevisiae* when primarily di- and tripeptide substrates were used. (This paper was submitted by B.R. to the City University of New York in partial fulfillment of the requirements for a Ph.D. degree.)

MATERIALS AND METHODS

Chemicals. All peptides and amino acid derivatives were in the L form except where noted. Most substrates used in these experiments were products of either Bachem Inc., Mann Research Laboratories, or Sigma Chemical Co. Acetyl (Ac)-Met-Met-Met and Ac-Met-Met were prepared by reacting the free peptides with acetic anhydride, whereas Met-Met-Met-methyl ester was prepared from tertiary butyloxycarbonyl-Met-Met-Met-methyl ester by cleavage of the tertiary butyloxycarbonyl group with HCl in methanol. The detailed experimental procedures are given elsewhere (20). The polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad Laboratories, and the peptidase staining reagents were from Sigma Chemical Co. All other reagents were of analytical quality.

Peptide purity determination. All peptides and their derivatives were shown to be homogeneous by thin-layer chromatography or high-voltage paper electrophoresis or both. Thin-layer chromatography was performed on silica gel plates in butanol–acetic acid–water (4:1:1). The peptides were detected by using iodine vapors or ninhydrin. High-voltage paper electrophoresis was carried out in a model LT-36 electrophoresis tank with E.C. 123 coolant and an HV-5000 power supply (Savant Instruments, Inc., Hicksville, N.Y.). Pyridine–acetate buffer (pH 3.5) was prepared from glacial acetic acid–pyridine–water (10:1:189, vol/vol). For some peptides a pH 1.9 buffer of formic acid–glacial acetic acid–water (6:24:270, vol/vol) was used. Peptides were applied to Whatman 3 MM paper and run at a gradient of 37 V/cm for 1.5 to 3 h. After electrophoresis, the paper was dried, dipped in a solution of ninhydrin (0.5%, wt/vol) in 95% aqueous acetone, and developed in a heated and ventilated chromatography oven. The ninhydrin stains were fixed by dipping the paper in a solution of 1% $Cu(NO_3)_2$ and 0.2% HNO_3 in acetone–ethanol (2:1).

Preparation *S. cerevisiae* Z1-2D cell-extract. Strain Z1-2D, a leucine–lysine auxotroph of *S. cerevisiae*, was received from Nasim Khan, Brooklyn College, City University of New York, New York, N.Y.

This haploid strain was derived from a cross between strains KC-372 (R. Mortimer, University of California, Berkeley) and 1323-1B (D. C. Hawthorne, University of Washington, Seattle). Cells were grown at 30°C with shaking in a minimal growth medium containing 0.67% yeast nitrogen base, leucine (60 μg/ml), and lysine (20 μg/ml). The cells were harvested at late log phase by centrifugation and disrupted by grinding with glass beads in an Omni-mixer. Cell extracts were dialyzed against 50 mM Tris buffer, pH 8.3. The detailed procedures for both cell growth and the preparation of cell extracts are given elsewhere (14).

Electrophoresis. Polyacrylamide gels (0.5 by 6.5 cm; 7% acrylamide, 0.186% bisacrylamide) were prepared in a manner similar to that described by Davis (4) with the following exceptions: sample and stacking gels were omitted, and the running gel was 50 mM in Tris buffer, pH 8.3. All electrophoretic runs were carried out by using a Bio-Rad model 151 tube gel electrophoresis unit, with cooling supplied by a circulating ice water bath. Gels were subjected to pre-electrophoresis by using running gel buffer in the buffer tanks for 2 h at 3 mA/tube and allowed to cool at 4°C either for 3 h or overnight. Protein samples for electrophoresis were prepared as follows. To 100 μl of dialyzed crude cell extract in running gel buffer were added 50 μl of distilled water and 15 μl of a 30% (vol/vol) glycerol solution containing 0.0004% bromophenol blue. To the top of each gel was applied 25 μl of the above mixture, which contained approximately 300 μg of protein as determined by the method of Lowry et al. (13). Both upper and lower buffer reservoirs contained Tris–glycine buffer (0.6 g of Tris and 2.88 g of glycine per liter, pH 8.3). The upper buffer reservoir (approximately 600 ml) contained 4 to 6 drops of a 1% aqueous solution of bromophenol blue. Electrophoresis was performed at 1 mA/tube, and the electrophoresis was terminated when the dye marker neared the end of the gel. The gels were immediately removed from their glass tubes and stained for either peptidase activity or protein.

Protein staining. The gels were stained for protein with Coomassie brilliant blue by the method of Chrambach et al. (2). Although this procedure is rapid (1 h) and does not require extensive destaining, it was found that, if the gels were allowed to stain overnight at room temperature and then destained slowly in 10% trichloroacetic acid, the band intensities increased and minor bands became visible.

Peptidase staining. Staining for peptidase activity was performed by the method of Lewis and Harris (12). A solution containing phosphate buffer, peptide, L-amino acid oxidase, horseradish peroxidase, *o*-dianisidine dihydrochloride, and manganous chloride was mixed with an equal volume of agar (2%) at 50°C and poured over a polyacrylamide gel placed in a test tube. Peptidase activity was determined within 90 min by the development of brown bands in the agar overlay. Only compounds capable of yielding L-amino acids upon hydrolysis which are substrates for L-amino acid oxidase may be used. Hydrolytic activity toward leucyl-2-naphthylamide and leucyl-*p*-nitroanilide was determined as described by Miller and MacKinnon (18).

RESULTS AND DISCUSSION

The results of the polyacrylamide gel electrophoresis-coupled peptidase staining method are tabulated in Table 1. A photograph of an actual staining pattern for the hydrolysis of Leu-Leu-Leu and a schematic representation of the same pattern are shown in Fig. 1. The true activity band widths are more closely represented by those shown in the schematic. The discrepancy between the widths in the photograph and those depicted in the schematic is due to differences in the times required for each activity band to develop. Bands which appear first tend to diffuse while other bands are still developing. There are at least four distinct aminopeptidase activities, as demonstrated by their ability to cleave several unblocked tripeptides, Met-Met-Met-methyl ester, and Leu-Gly-NH_2, and their inability to cleave Ac-Met-Met-Met and Ac-Phe. The only

TABLE 1. *Peptidase specificity patterns*[a]

Substrate[b]	Peptidase activity in gel band:			
	1	2	3	4
Met-Met-Met	+	+	+	+
Met-Gly-Met	+	+	+	+
Leu-Leu-Leu	+	+	+	+
Met-Ala-Met	+	+	+	+
Met-Gly-Gly	+	−	−	+
Gly-Leu-Gly	+	−	+	−
Gly-Met-Gly	+	−	+	−
Ac-Met-Met-Met	−	−	−	−
Met-Met-Met-OMe	+	+	+	+
Met-Met	−	−	+	−
Met-Leu	−	−	+	−
Leu-Met	−	−	+	−
Met-Gly	−	−	+	−
Gly-Met	−	−	+	−
Met-Ala	−	−	+	−
Leu-Leu	−	−	+	−
Leu-Gly	−	−	+	−
Gly-Leu	−	−	+	−
Met-Pro	−	−	−	−
Pro-Met	−	−	+	−
Leu-Pro	−	−	−	−
Pro-Leu	−	−	+	−
Ac-Met-Met	−	−	−	−
Ac-Gly-Leu	−	−	−	−
Ac-Phe	−	−	−	−
Leu-Gly-NH_2	+	+	−	−
Leu-NH_2	−	−	−	−
Z-Phe-Leu	−	−	−	−
Z-Leu-Leu	−	−	−	−
L-Leu-D-Leu	−	−	−	−
D-Leu-L-Leu	−	−	−	−

[a] Cell extracts of *S. cerevisiae* Z1-2D were electrophoresed in polyacrylamide gels and stained for peptidase activity as described in the text. The formation of brown bands in the agar overlay was indicative of a positive response. Bands 1 through 4 correspond to those shown in Fig. 1.

[b] Abbreviations: OMe, methyl ester; Z, benzyloxycarbonyl.

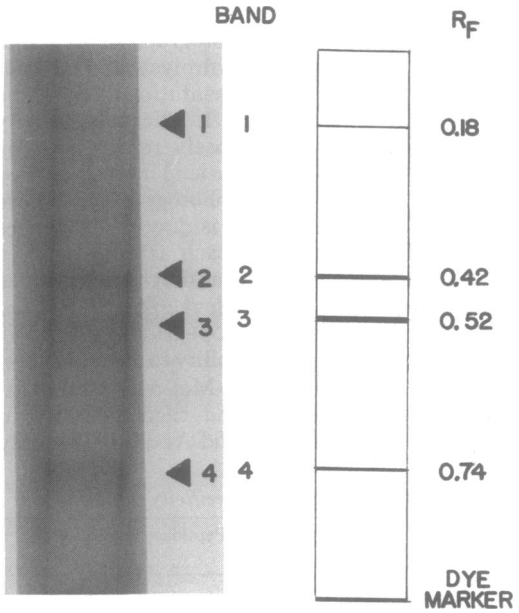

FIG. 1. *Peptidase staining pattern for Leu-Leu-Leu. R_f = Distance migrated by the activity band/ distance migrated by the dye marker.*

dipeptidase activity found resides in band 3. Since activity toward both dipeptides and tripeptides was found in band 3, we suspected that this band was composed of at least one aminopeptidase and one dipeptidase. The dipeptidase and aminopeptidase were subsequently separated by hydroxylapatite chromatography. Both activities had the same R_f as band 3 when examined by the enzyme-coupled activity stain on polyacrylamide gels (B. Rose, unpublished data).

The peptidase activities in bands 1 through 4 each cleaved Met-Met-Met, Met-Gly-Met, Leu-Leu-Leu, and Met-Met-Met-methyl ester, thus demonstrating overlapping substrate specificities. More important, however, is the fact that they could be distinguished from one another by their electrophoretic mobilities and their actions on Met-Gly-Gly, Gly-Met-Gly, and Leu-Gly-NH_2. The peptidase activity found in band 1 cleaved all of these substrates and seemed to have the broadest specificity. The aminopeptidase activities in bands 2, 3, and 4 each cleaved only one of these substrates, and in each case it was a different one. It is believed that each of these peptidase activities belongs to a different and distinct aminopeptidase.

The response toward Gly-Met-Gly represents a very interesting case. In order for this peptide to yield an activity band, it must undergo a two-step reaction because glycine is not a substrate for the L-amino acid oxidase contained in the activity staining mixture. Therefore, Gly-Met-Gly must first be broken down to glycine and a dipeptide, followed by cleavage of the dipeptide to liberate methionine. In light of this, one must explain the positive result for bands 1 and 3. For band 3, the idea of two separate hydrolysis reactions by two different peptidases is plausible since this band contains both amino- and dipeptidase activities. However, this does not explain the activity observed for band 1, which under assay conditions did not cleave any of the dipeptides tested, including Met-Gly. It is proposed that a positive response for Gly-Met-Gly from band 1 involves a two-step hydrolysis reaction by the same aminopeptidase. The first step involves cleavage of the tripeptide to glycine and Met-Gly, followed by hydrolysis of the dipeptide by the aminopeptidase itself. It is well known that yeast aminopeptidases are capable of cleaving dipeptides (5, 15, 17). The negative response of the aminopeptidases here to the dipeptides tested may be due in part to the assay conditions employed and the time required for the assay. The unexpected positive response of activity band 1 toward Gly-Met-Gly may be explained by assuming that the active sites for di- and tripeptide hydrolyses are identical. Cleavage of Gly-Met-Gly produces Met-Gly at the active site, and then the dipeptide, which is present at a high local concentration and in correct position for hydrolysis, is cleaved to liberate glycine and L-methionine. The L-methionine released is responsible for the positive response observed. Similar results were also observed with Gly-Leu-Gly. To check our hypothesis for the activity in band 1, the dipeptides Met-Gly and Leu-Gly were used in the peptidase assay at 5, 10, and 30 times the standard concentration (5 mg of peptide per assay). However, there was still no cleavage of either dipeptide by the activity in band 1. These results do not necessarily negate our hypothesis since it may not be possible to achieve both the high concentration and proper orientation of the dipeptide in the active site of the enzyme by merely supplying it at high concentrations in the assay mixture.

A single dipeptidase activity was found for *S. cerevisiae* Z1-2D and resides in band 3. All free dipeptides which were tested except Met-Pro, Leu-Pro, L-Leu-D-Leu, and D-Leu-L-Leu were cleaved to their constituent amino acids. The failure of the dipeptidase to cleave X-Pro and L-, D-, or DL-dipeptides was due to requirement of this enzyme for an amide bond and its stereospecificity. This specificity has also been observed for several dipeptidases in bacteria (11, 19, 23) and for a purified yeast dipeptidase (24).

All blocked dipeptides tested were not cleaved by the activity in band 3, illustrating the requirement for both a free amino and a carboxy terminus. This specificity is expected for true dipeptidase character (21). The above observations have been confirmed on gels and in solution for a purified form of this dipeptidase (Rose, unpublished data).

No carboxypeptidase activity was found under the standard assay conditions described above. It is well known, however, that cell extracts of *S. cerevisiae* contain carboxypeptidase activity, which must be activated before assay (10). Activation of cell extracts and demonstration of carboxypeptidase activity toward benzyloxycarbonyl-Phe-Leu was accomplished in solution by using the procedures described by Hayashi (7) and Rohm (24). The activated cell extract was then dialyzed against the Tris buffer used for preparing protein samples for electrophoresis, and the assay was carried out. However, no carboxypeptidase activity was observed on the polyacrylamide gels. The exact reason for the discrepancy between solution and gel assays is not known. It is suspected, however, to be a pH-related problem since yeast carboxypeptidase activity toward amine terminus-blocked dipeptides is usually maximum at pH values below 7.0 (8, 21).

Matile et al. (16) reported four aminopeptidase activities for *S. cerevisiae* cells grown to stationary phase. These activities were measured by starch gel electrophoresis coupled with an activity stain in which leucyl-2-naphthylamide, a nonpeptide substrate, was used in the presence of 3 mM Co^{2+}. The use of substrates other than peptides to monitor peptidase activity may lead to erroneous conclusions. Such a case was recently cited by Payne (22) regarding the alleged occurrence of an inducible periplasmic aminopeptidase in *Escherichia coli* based on activity toward *p*-nitroanilide and 2-naphthylamide amino acid derivatives. Although a recent report by Lazdunski and coworkers (1) shows that a number of di- and higher peptides are cleaved by this periplasmic enzyme, extremely high substrate concentrations were necessary for the cleavage of dialanine. It is thus not clear whether activity toward amino acid nitroanilides always correlates with peptidase activity.

To investigate this point further, we tested the activity of cell extracts of *S. cerevisiae* Z1-2D against leucyl-2-naphthylamide and leucyl-*p*-nitroanilide in the presence and absence of 3 mM Co^{2+}, using the procedure of Miller and MacKinnon (18). Only two activity bands, having R_f values similar to those of bands 1 and 2 as determined by the polyacrylamide gel electrophoresis-coupled activity staining procedure, were observed. These results suggest that at least two of the aminopeptidase activities found in this study do not cleave amino acid derivatives. It is reasonable to conclude, therefore, that some of the activities reported by Matile and coworkers (16) differ from the aminopeptidase activities in bands 1 through 4 (Fig. 1).

In conclusion, this study shows that at least four independent aminopeptidases, one dipeptidase, and an as-yet-unspecified carboxypeptidase activity are present in a leucine–lysine double auxotroph of yeast. We are currently characterizing the dipeptidase and are attempting to investigate its function in the transport and metabolism of peptides by yeast.

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