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NOVEL LEU-LYS-SPECIFIC PEPTIDASE (LEULYSIN) PRODUCED BY GEL-ENTRAPPED YEAST CELLS

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Summary: Saccharomyces cerevisiae cells entrapped with a neutral hydrophilic photo-crosslinkable resin prepolymers specifically excreted into a cultured medium a new type of a peptidase, which cleaved Leu-Lys bond of α-mating factor. The enzyme was purified by membrane filtration followed by ion exchange chromatography and gel filtration chromatography. The purified enzyme showed a strict substrate specificity on internal Leu-Lys bond. Leu-Lys bond near terminus of a molecule and Leu-X and X-Lys bonds examined so far were not hydrolyzed by the enzyme.

Saccharomyces cerevisiae strain X-2180 1B (α-type) is known to produce a pheromone, α-mating factor (1), which is a peptide having an amino acid sequence of Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (2). Simultaneously the yeast cells show an activity to cleave peptide bonds of α-mating factor. This degrading activity has been considered to be ascribable to several kinds of proteolytic enzymes excreted from the cells (3). In spite of many efforts, however, the properties of the proteolytic enzymes are unclear because of their low amounts in the cultured medium.

In the course of the study on the production of α-mating factor by gel-entrapped yeast cells (4), we have found that S. cerevisiae cells entrapped in an appropriate polymer gel produced extracellularly relatively high levels of the proteolytic enzymes.

This paper describes the purification of a novel Leu-Lys-specific peptidase from the cultured medium of the entrapped yeast cells and some properties of the enzyme, which has been named "Leulysin".

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Materials and Methods

Yeast Strain and Cultivation Cells of Saccharomyces cerevisiae strain X-2180 1B (α-type) were immobilized in a synthetic polymer gel and cultivated for 24 h in a 2-liter jar fermentor containing 2 liter of Burkholder medium at 30 °C under aerobic condition (aeration rate, 1 VVM).

Immobilization of Cells About 2 g of fresh cells were immobilized aseptically with 1 g of a neutral hydrophilic photo-crosslinkable resin prepolymer, ENT-2000 (5), as described previously (6). The gel formed (thickness, about 0.5 mm) was cut into small pieces (about 5 x 5 mm) and used for the production of Leu-Lys-specific peptidase at a concentration of 35 g of immobilized cell preparation per liter of medium.

Enzyme Assay The enzyme and α-mating factor (5 μg) were incubated at 30 °C in 0.4 ml of 50 mM acetate buffer (pH 4.0) for 6 to 24 h. Amounts of residual α-mating factor and the degradation products were assayed by using a high performance liquid chromatography (Water Co., analytical separation system) under the following conditions: Column, μ-Bondapak C_{18} column; mobile phase, acetonitrile in 0.1 % trichloroacetic acid (about pH 2.0) (gradient elution of 5 - 60 % for 30 min); flow rate, 1.5 ml/min; detector, ultraviolet monitor (214 and 280 nm).

Chemicals A photo-crosslinkable resin prepolymer, ENT-2000 (a derivative of poly(ethylene glycol)-2000) was donated by Kansai Paint Co., Tokyo, Japan. α-Mating factor and other synthetic peptides were purchased from Peptide Institute Inc., Osaka, Japan. All other chemicals were also obtained from commercial sources.

Results and Discussion

When the ENT-2000-entrapped cells of Saccharomyces cerevisiae strain X-2180 1B were grown aerobically, the amount of α-mating factor in the cultured medium reached the maximum during the exponential growth phase and then decreased to become nil in the stationary phase. The enzyme activity of α-mating factor degradation in this cultured medium was about 0.2 $nmol.h^{-1}.ml^{-1}$ when measured in 50 mM acetate buffer (pH 4.0) at 30 °C. This value was far higher than that in the cultured medium of the free yeast cells.

The cultured medium (2,000 ml) was passed through a microfilter (0.22 μm) (Millipore Co.) to remove the cells, and concentrated to about 30 ml by ultrafiltration with a membrane PU-10 (Amicon Co.). The concentrated solution was dialyzed for 24 h at 4 - 8 °C against distilled water by changing water three-times. After dialysis, precipitates formed were removed by centrifugation and the crude enzyme was lyophilized. The lyophilized enzyme was dissolved in 10 ml of 10 mM potassium phosphate

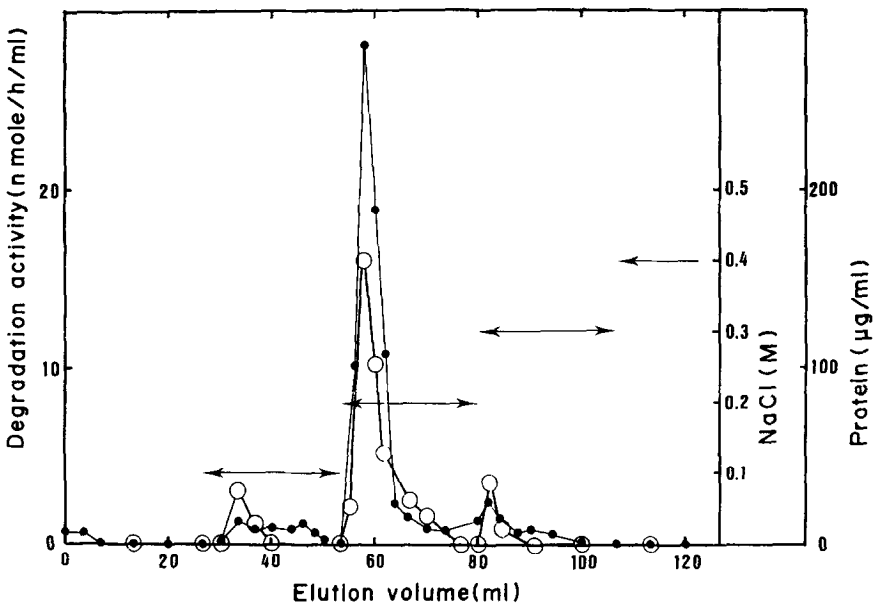

Fig. 1. DEAE-Sephacel column chromatography of proteolytic enzymes. Experimental methods are described in the text. ○, α-Mating factor-degrading activity (expressed as the amount of 1-6 peptide formed); ●, protein; ←→, NaCl concentration.

buffer (pH 7.0) and applied onto a CM-Sepharose column (1.0 x 13 cm) equilibrated with the same buffer. Active fraction passed through the column was applied onto a DEAE-Sephacel column (1.0 x 13 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.2), and the column was washed with 50 ml of the same buffer. The enzyme was eluted with a discontinuous gradient of NaCl in the Tris-HCl buffer. α-Mating factor-degrading activities were mainly recovered in the fractions of 0.1 - 0.3 M NaCl (Fig. 1). When each fraction was examined as to α-mating factor degradation, the 0.2 M NaCl fraction gave only two degradation products, which were assumed to be 1-6 peptide and 7-13 peptide on high performance liquid chromatography, while other fractions produced several kinds of peptides. These results indicated that Leu-Lys-specific peptidase was eluted at 0.2 M NaCl and other peptidases at different concentrations of NaCl.

The 0.2 M NaCl fraction was applied onto a Sephacryl S-500 column (1.0 x 45 cm) equilibrated with 100 mM acetate buffer (pH 4.0) and the enzyme was eluted with the same buffer at a flow rate of 0.15 ml/min.

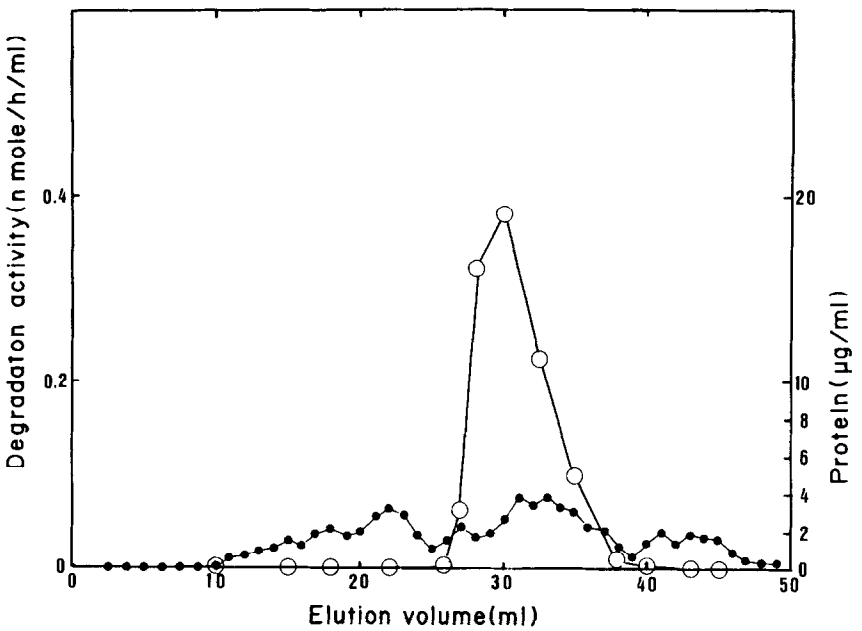

Fig. 2. Sephacryl S-500 column chromatography of proteolytic enzymes. Experimental methods are described in the text. ○, α-Mating factor-degrading activity (expressed as the amount of 1-6 peptide formed): ●, protein.

When each fraction was examined for the α-mating factor degradation, fractions No. 28 - 31 showed the high activity (Fig. 2). On sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis, the enzyme preparation gave only two protein bands whose molecular masses were estimated to be 55,000 and 63,000 daltons, respectively (Fig. 3). Molecular mass of the native form of Leu-Lys-specific peptidase was about 750,000 daltons (larger than thyroglobulin, 669,000 daltons) when measured by gel filtration on Sephacryl S-500 with 100 mM acetate buffer (pH 6.0) at a flow rate of 0.15 ml/min.

When the enzyme was incubated with α-mating factor for 6 h, the optimal reaction temperature was 37 °C. The highest activity of the enzyme was obtained at pH 4.0 in 100 mM acetate buffer.

The purified enzyme gave two degradation products from α-mating factor as mentioned above. These peptides were fractionated by high performance liquid chromatography (Fig. 4) and analyzed with a Hitachi Amino Acid Analyzer type 3. The results obtained indicated that one

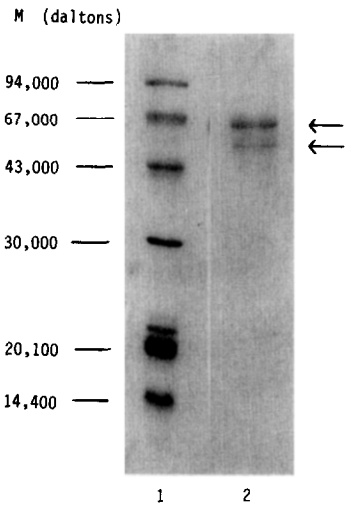

Fig. 3. Sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis of the purified proteolytic enzyme. Lane 1, marker peptides; lane 2, the purified enzyme. Marker peptides used are phosphorylase b (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (20,100 daltons), and α-lactalbumin (14,400 daltons).

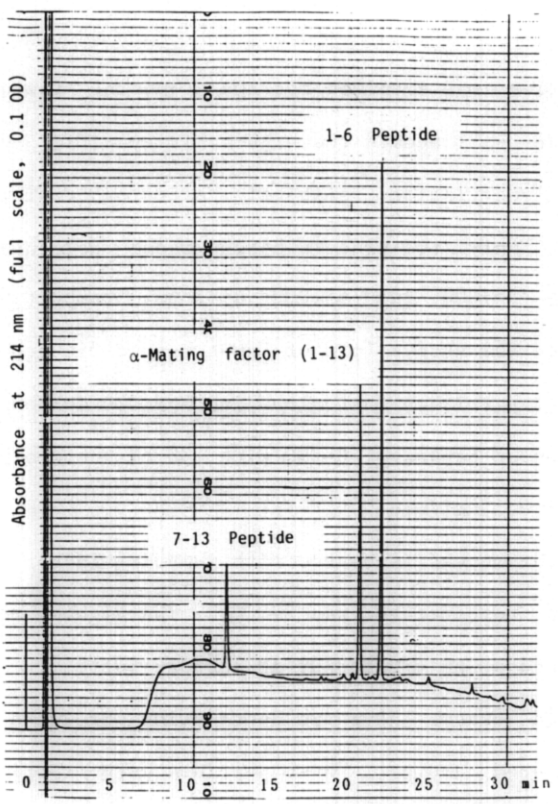

Fig. 4. Analytical elution profile of α-mating factor degradation products formed by the purified enzyme. Experimental methods are described in the text.

Table 1. Peptides Used as Substrates for Leu-Lys-Specific Peptidase

Peptides having internal Leu-Lys Bond	
α-Mating factor:	Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr
Dynorphin:	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Peptide having Leu-Lys bond at near N-terminus	
Mastoparan:	Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2
Peptides having internal X-Lys and/or Leu-X bond(s)	
Neurotensin:	Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Tuftsin:	Thr-Lys-Pro-Arg
γ-Endorphin:	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu
α-HANP:	Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr
Oxytocin:	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
Corticotropin-releasing hormone:	Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-Ile-NH_2

peptide eluted at 14.8 min was Lys-Pro-Gly-Gln-Pro-Met-Tyr (7-13 peptide) and the other eluted at 24.3 min Trp-His-Trp-Leu-Gln-Leu (1-6 peptide). This fact revealed the specificity of the enzyme on Leu-Lys bond.

To clarify the substrate specificity of the enzyme, several peptides were subjected to the enzymatic degradation (Table 1). Of these peptides examined α-mating factor and Dynorphin, which have internal Leu-Lys bond, were found to be susceptible to the enzymatic reaction, while Mastoparan having Leu-Lys bond near the N-terminus was not degraded at all. In addition, X-Lys and/or Leu-X bonds in α-mating factor and other peptides examined were not cleaved at all by the enzyme. The results indicate clearly that the enzyme purified from the cultured medium of immobilized S. cerevisiae cells is a unique and novel peptidase specific to the internal Leu-Lys bond. Therefore, we named this enzyme "Leulysin".

Such the specific peptidase will be of use for processing of various physiologically active proteins and peptides, and also for study of protein structures.

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