Purification and Properties of Extracellular Carboxyl Proteinase Secreted by *Candida pulcherrima*

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An extracellular proteinase secreted by *Candida pulcherrima* KSY 188-5 was purified about 60-fold to electrophoretical homogeneity from its culture supernatant, by ammonium sulfate fractionation, anion-exchange chromatography, and gel filtration. The proteinase had a molecular weight of approximately 36,500 and an isoelectric point of pH 4.7. The enzyme had an optimum pH of around 2.5-3.5 for activity and 3.0-5.0 for stability. The optimum temperature was around 45°C at pH 3.0. The enzyme showed a broad substrate specificity for a variety of proteins to hydrolyze casein, BSA, hemoglobin, keratin, and collagen. Among several proteinase inhibitors, pepstatin A completely abolished the enzyme activity, indicating that the extracellular proteinase from *C. pulcherrima* KSY 188-5 was classified in the group of carboxyl proteinases.

Extracellular proteinases secreted by yeasts have been isolated and examined by several researchers with regard to their enzymatic and immunological properties and the physiology of induction of their secretion. Most of these studies are on the enzymes from *Candida albicans*. This is because *C. albicans* is of foremost importance as an opportunistic pathogen in humans, and among possible virulence factors in its pathogenicity the proteinases have gained substantial importance. The ability of other *Candida* spp. to secrete extracellular proteinases and their immunological correlation have also been reported. However, studies with respect to enzymatic properties of these proteinases from yeasts other than *C. albicans* are limited.

Kodama et al. screened many strains of yeasts from tree exudates and isolated *Candida pulcherrima* (strain KSY 188-5) as having characteristics of secreting a proteolytic enzyme active in acidic pH. In this work we describe the purification and some properties of the extracellular proteinase secreted by *C. pulcherrima* KSY 188-5.

**Materials and Methods**

*Microorganism.* *Candida pulcherrima* strain KSY 188-5, which secretes a proteinase in broth, was isolated by Kodama from a tree exudate.

The strain was maintained on an agar slant of YM medium (glucose, 10 g/l; peptone, 5 g/l; malt extract, 3 g/l; and yeast extract, 3 g/l) at 4°C.

*Fermentation.* The cells of the agar slant culture were inoculated into a 200-ml shaking flask containing 100 ml of YM medium, and grown at 30°C for 24 h on a reciprocal shaker. This seed culture was then transferred to 1.5 liters of a semi-synthetic medium (glucose, 10 g/l; KH₂PO₄, 1 g/l; MgSO₄·7H₂O, 0.2 g/l; yeast extract, 0.1 g/l; and casein, 4 g/l) with an inoculum size of 1% (v/v). Culture was done in a jar fermentor MD-250 (L. E. Marubishi Co.) with a 6-blade turbine impeller (60 x 13 mm) and 4 baffle plates, at 30°C for 24 h. The culture medium was aerated through a ring sparger at 1.5dm³/min and agitated at 60 rpm. The pH was maintained at 3.0 by adding 1 N NaOH or 1 N HCl.

*Purification.* All the procedure were done at 4°C. Fresh culture broth was centrifuged at 900 x g for 30 min to remove cells. Ammonium sulfate (341 g) was added to 1300 ml of the supernatant (45% saturation), and the mixture was centrifuged at 2000 x g for 60 min. The supernatant was discarded and the pellet was dissolved in 25 ml of 20 nm citrate buffer (pH 4.8) containing 1 nm EDTA. The resultant solution was dialyzed against 20 nm citrate buffer (pH 6.8) containing 1 nm EDTA and put on a column (ø 3 x 32.5 cm) of DEAE-Toyopearl 650M (Tosoh), which had been equilibrated with the same buffer. The column was washed with 300 ml of the buffer, and the proteinase was eluted with a linear gradient of 1 l of 0 to 1 M NaCl in the buffer. The active fractions were combined and the enzyme was precipitated by adding solid (NH₄)₂SO₄ to 95% saturation. The resultant precipitate was dissolved in 4 ml of 20 nm citrate buffer (pH 4.8) containing 20 nm NaCl, and the solution was put on a gel filtration column (ø 2.5 x 50 cm) of HW-55 Toyopearl (Tosoh), which had been equilibrated with the same buffer. Fractions containing proteinase activity were combined and stored as purified enzyme at 0°C.

*Protein assay.* Proteins were measured by the BCA method with bovine serum albumin (BSA) as a standard protein. Proteins eluted from the columns were monitored by change in absorbance at 280 nm.

*Enzyme assay.* Unless otherwise stated, the proteinase activity was measured essentially by the casein-Folin method. The assay mixtures contained, in a final volume of 3.0 ml, 1% casein, 1.0 ml of McIlvaine buffer solution (pH 3.0), and a suitable amount of the purified proteinase. After the mixtures were incubated at 37°C for 30 min, the reaction was stopped by addition of 3.0 ml of 0.4 M trichloroacetic acid. To a 0.5-ml sample of the supernatant obtained by centrifugation at 2000 x g for 30 min was added the phenol reagent. The solution was kept at 37°C for 30 min and the absorbance at 660 nm was measured. One unit (PU) of proteinase activity was defined as the activity required to hydrolyze casein to give non-proteinous peptides whose coloration by the phenol reagent...
treatment was identical to that of 1 μg of tyrosine per minute.

**Electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) with or without sodium dodecyl sulfate (SDS) was done at a constant current of 5 mA on vertical slab gels of 15% and 12.5% polyacrylamide, with a stacking gel of 5% polyacrylamide gel, essentially as described by Laemmli.\(^{19}\) MW-markers (Kanto Chemical Co.), which consisted of the monomer, dimer, trimer, and pentamer of cytochrome c from horse heart, were used for molecular weight standards. The gel was then stained with 0.5% Coomassie brilliant blue R-250 in 50% methanol-10% acetic acid solution.

**Isoelectric focusing.** Isoelectric gel electrophoresis was done with Ampholine (Sigma) carrier ampholite solution, using 4% polyacrylamide gel in capillary glass tubes (φ 1.3 × 80 mm) for 2 h at a constant voltage of 300 V following for 30 min at a constant current of 1 mA. After the electrophoresis, one of the tubes was divided into 10-mm-long pieces, crushed, and immersed in 1 ml of distilled water. After 24 h, the pH of the solution was measured by a pH meter. The gel of the other was stained with Coomassie Brilliant Blue R-250.

**Amino acid analysis.** The enzyme prepared was hydrolyzed with 6 N HCl in vacuo at 110°C for 24, 36, and 48 h. The amino acid composition was analyzed by a Shimadzu LC-10A System liquid chromatograph. The contents of Ser, Thr, and Tyr were estimated by extrapolation of the data of 24, 36, and 48 h hydrolysis to zero time.

**Results**

**Fermentation**

The extracellular proteinase of *C. pulcherrima* KSY 188-5 was produced in a semi-synthetic medium containing casein protein. This medium stimulated the secretion of the enzyme about three times more than YM medium. After the fermentation, the culture supernatant of the semi-synthetic medium contained 30 PU/ml of proteinase activity. This was similar to those obtained in the cultures of *C. albicans*.\(^{40}\) Casein in the semi-synthetic medium appeared to induce the secretion of the proteinase by the yeast. The proteinase secretion was decreased to a tenth by the replacement of casein by (NH₄)₂SO₄ as a nitrogen source in the semi-synthetic medium (data not shown).

The YM medium gave the maximum growth yield of *C. pulcherrima* KSY 188-5 of about 2.5 mg dry cells/ml. Therefore, the seed culture was made with the YM medium.

**Purification**

The extracellular proteinase was purified from the culture supernatant of *C. pulcherrima* KSY 188-5. The results for a typical purification are summarized in Table 1. The (NH₄)₂SO₄ fractionation removed major proteinous compounds and much raised the specific activity of the enzyme. Dialysis also raised the specific activity. This is because the culture broth contained a lot of proteinous compounds or peptides of low molecular weight, which were degraded from casein by the action of the secreted proteinase. On DEAE ion-exchange chromatography, the maximal activity of the proteinase was observed at the conductivity of 25 mS/cm (Fig. 1). After Toyopearl HW-55 gel filtration, the enzyme was purified 58.6-fold with a specific activity of 1343 PU/mg-protein and gave a single band on PAGE with staining by Coomassie brilliant blue R-250 (data not shown). The purified enzyme could be stored at 0°C without loss of activity for at least 2 weeks.

**Molecular properties**

SDS–PAGE showed that the final preparation of the extracellular proteinase migrated at the position corresponding to a molecular mass of 36,5 kDa (Fig. 2). The gel filtration gave a molecular mass of 36 kDa. These data indicated that the enzyme is a monomeric protein. Isoelectric focusing also gave a single protein band with a pI of 4.7.

Amino acid analysis showed that the extracellular proteinase was composed of 289 amino acid residues (Asp, 47; Thr, 21; Ser, 36; Glu, 25; Pro, 8; Gly, 23; Ala, 23; Val, 18; Ile, 13; Leu, 23; Tyr, 22; Phe, 13; His, 4; Lys, 6; Arg,

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**Table 1.** Purification of Extracellular Proteinase from *C. pulcherrima* KSY 188-5

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (PU)</th>
<th>Specific activity (PU/mg)</th>
<th>Yield activity (%)</th>
<th>Ratio (–)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>1333</td>
<td>30,477</td>
<td>22.9</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>54.1</td>
<td>11,185</td>
<td>206.7</td>
<td>36.7</td>
<td>9.0</td>
</tr>
<tr>
<td>Dialysis</td>
<td>35.7</td>
<td>10,877</td>
<td>304.7</td>
<td>35.7</td>
<td>13.3</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>12.5</td>
<td>10,231</td>
<td>818</td>
<td>33.6</td>
<td>35.7</td>
</tr>
<tr>
<td>Toyopearl HW-55</td>
<td>7.1</td>
<td>9,538</td>
<td>1343</td>
<td>31.1</td>
<td>58.6</td>
</tr>
</tbody>
</table>

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**Fig. 1.** DEAE-Toyopearl Chromatogram of 0-45% Ammonium Sulfate Fraction.

Fractions were examined for proteinase activity (○) under the standard assay conditions and for protein (●) by absorbance at 280 nm. The dots (·) indicate conductivity.
5; Cys, 2), based on the molecular mass of 36.5 kDa. Asp, Ser, and Glu residues were abundantly contained, but there was no Met residue.

**Enzymatic properties**

The effects of pH on the proteinase activity were examined in buffer solutions of various pH’s. As Fig. 3A shows, the optimum pH for the proteinase reaction is around at pH 2.5 to 3.5 with casein as a substrate at 37°C. The pH stability of the enzyme was also examined. After the enzyme was incubated in buffer solutions of various pH’s at 37°C for 12 h, remaining activities were measured at pH 3.0. The enzyme is stable at pH’s between 3.0 and 5.0, but extremely unstable above pH 6.5 (Fig. 3B).

The effects of temperature on the extracellular proteinase were investigated. The optimum temperature for the enzyme reaction at pH 3.0 was around 45°C (Fig. 4A). The thermal stability of the enzyme was examined by incubation at various temperatures at pH 3.0 for 30 min before the standard assay at pH 3.0 at 37°C. As Fig. 4B shows, the enzyme was stable up to 45°C, and above the temperature the stability decreased sharply.

The substrate specificity of the extracellular proteinase
from *C. pulcherrima* KSY 188-5 was examined with casein, BSA, bovine hemoglobin, collagen, and keratin as substrate proteins. From the results shown in Fig. 5, the proteinase was found to have a broad protein substrate specificity to hydrolyze these proteins. Apparent low activities with keratin and collagen are expected to be due to their insoluble character in the buffer solution. The maximum activity was obtained with casein protein.

The effects of several proteinase inhibitors were examined. The extracellular proteinase was inhibited by pepstatin A (Fig. 6A), but not by iodoacetamide, dithiothreitol, 2-mercaptoethanol, disopropyl fluorophosphate, and EDTA (data not shown), when casein was used as substrate. These findings indicate that the enzyme is classified in a group of carboxyl proteinases. Of synthetic inhibitors of carboxyl proteinases, however, diazoacetyl-dl-norleucine methyl-ester (DAN) showed no inhibition against the enzyme with a final molar ratio of the enzyme: DAN: Cu^{2+} = 1:146:292 in a 50 mM citrate buffer (pH 5.6) (Fig. 6B), though another synthetic inhibitor, 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), inactivated the enzyme in a 50 mM citrate buffer (pH 4.8) (Fig. 6C).

**Discussion**

The properties of the purified enzyme are summarized in Table II with those of the proteinase from *C. albicans* for comparison. The properties with respect to pH seem to be in the range of those of usual carboxyl proteinases. In molecular and enzymatic aspects, the proteinase from *C. pulcherrima* KSY 188-5 was quite similar to the ones from *C. albicans*.

The extracellular proteinase from *C. pulcherrima* KSY 188-5 obviously belongs to the class of carboxyl proteinases since it is completely inhibited by pepstatin A, a potent inhibitor for carboxyl proteinases, but not by other inhibitors specific for serine, thiol, and metallo proteinases. The extracellular proteinase from *C. albicans* is insensitive to the synthetic inhibitors DAN and EPNP, but the proteinase from *C. pulcherrima* KSY 188-5 was inhibited by EPNP, but not by DAN. There is a substantial amount of information for inhibition of several other carboxyl proteinases,16–19 which has been mainly gained during the design of inhibitors for candidosis and for the retroviral proteinases of HIV. However, the relation between the differences in inhibitor sensitivity of carboxyl proteinase family and chemical and conformational properties in the active site of the enzymes is quite complicated and remains to be resolved.

The extracellular proteinase from *C. pulcherrima* KSY 188-5 had a broad protein substrate specificity to hydrolyze casein, BSA, bovine hemoglobin, collagen, and keratin, as had the proteinases from *C. albicans*. The enzymes from *C. albicans* are recognized to be a potential virulence factor that may facilitate the colonization and invasion of the

![Fig. 6. Effects of Carboxyl Proteinase Inhibitors on Proteinase from *C. pulcherrima* KSY 188-5.](image)

**Table II. Comparison of Extracellular Proteinases of *C. pulcherrima* KSY 188-5 and *C. albicans***

<table>
<thead>
<tr>
<th>Properties</th>
<th>KSY 188-5</th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>2.5–3.5</td>
<td>3.0–4.5</td>
</tr>
<tr>
<td>pH sensitivity</td>
<td>6.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>37,000</td>
<td>41,500 (44,000)*</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.7</td>
<td>4.5</td>
</tr>
<tr>
<td>Thermolability</td>
<td>60°C for 30 min</td>
<td>100°C for 10 min</td>
</tr>
<tr>
<td>Inhibition by</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pepstatin A</td>
<td>+</td>
<td>+*</td>
</tr>
<tr>
<td>DAN</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EPNP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Substrate reactivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BSA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Keratin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collagen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Abundant amino acids</td>
<td>Asp, Ser, Glu</td>
<td>Asp, Ser, Glu, Gly</td>
</tr>
</tbody>
</table>

* Data from ref. 7.
* The pH above which activity was irreversibly lost.
* Data from refs. 2 and 4.
* The temperature at which the enzymes were inactivated by incubation for indicated period.
yeast to skin. The broad protein substrate specificity is thus content with the role of the enzymes for \textit{C. albicans}. For \textit{C. pulcherrima} KSY 188-5, which was found in a tree exudate, the property of its extracellular proteinase is considered to be favorable to hydrolyze a variety of proteins in tree exudate and to supply amino acids and peptides as nutrition to the yeast.

The secretion of the proteinase from \textit{C. pulcherrima} KSY 188-5 was stimulated in a semi-synthetic medium containing casein as a nitrogen source. The same results were observed with \textit{C. albicans}. Banerjee\textsuperscript{20} reported that the proteinase secretion by \textit{C. albicans} was increased in the restrictive culture conditions that provided nitrogen solely in the form of medium- to high-molecular weight proteins. Though the molecular mechanism of the induction and secretion of proteinases in \textit{Candida} spp. incubated in the protein-supplemented medium remains to be investigated, the induction and secretion are expected to relate closely nutritive and bital requirements.

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References