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Purification and characterisation of a metallopeptidase of *Candida albicans*

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Summary. A novel aminopeptidase was purified by high performance liquid chromatography from a cytosoluble 100 000 g extract of *Candida albicans* on the basis of its ability to cleave L-arginine 7-amino-4-methylcoumarin. The purification factor was 36 and the yield was 20 %. The native enzyme had a mol. wt of 52 kDa as demonstrated by SDS-PAGE in the presence or absence of reducing conditions and exhibited an iso-electric point of 4.3. The aminopeptidase showed optimum activity at pH 7.2, a Michaelis constant of *c.* 50 μ M and a $V_{\max}$ at 19 mM AMC released/min/mg of protein for L-Arg-AMC. This enzyme was shown to cleave at low affinity L-leucine-7-amino-4-methylcoumarin as demonstrated by the spectrofluorimetric method. The enzyme was strongly inhibited by specific metallo-enzyme inhibitors—EDTA and *o*-phenanthroline. Furthermore, there is evidence that a similar or identical enzyme occurs in other *C. albicans* clinical isolates and other *Candida* spp.

Introduction

There is accumulating evidence of the involvement of yeast peptidases in host–micro-organism interactions, including digestion of exogenous proteins for nutritive purposes, invasion of host cells and tissues, and inactivation of host proteins that are detrimental to yeast survival.^{1,2}

Candida albicans is a dimorphic yeast that colonises mucosal surfaces of the gastrointestinal tract, genitourinary tract and the oral cavity. This organism has become an important pathogen in immunocompromised patients as well as in patients with various other underlying conditions that abrogate or impair immunity. Many peptidase activities have been detected in this yeast. Some of these enzymes have been purified and characterised.^{3–9} However, the biological functions of these peptidases are not known. An extracellular proteinase (SAP) was purified from the culture media of various strains of *C. albicans* by Rùchel,⁴ and was characterised as an aspartyl proteinase with broad substrate specificity. This glycoprotein had an M_r of 42–45 kDa and an activity in the pH range 2–7.^{2,4,10} An intracellular proteinase with optimum activity at pH 6.6⁹ and three cytoplasmic enzymes—a metal-ion-dependent aminopeptidase,⁵ a dipeptidase⁵ and a carboxypeptidase¹¹—have been

identified. Recently, two serine proteinases were detected from cytoplasmic extracts of *C. albicans* by SDS-PAGE with gels containing gelatin as proteinase substrate.¹²

In this investigation, a novel cytoplasmic *C. albicans* aminopeptidase activity was identified by means of highly sensitive substrates with methylcoumarin groups. The purification and characterisation of a 52-kDa metallopeptidase from cell-free extracts of *C. albicans* are described. *C. albicans* clinical isolates from urine, vagina and the mouth and other *Candida* species were examined for similar metallopeptidases.

Materials and methods

Materials

Substrates for peptidase including 7-amino-4-methylcoumarin derivatives (AMC), peptidase inhibitors—DTT, dithiothreitol; E64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; NEM, *N*-ethylmaleimide; phosphoramidon, *N*-(α -rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan; PMSF, phenylmethylsulphonyl fluoride; TLCK, *N*- α -*p*-tosyl-L-lysine chloromethyl ketone—and pre-stained mol. wt markers were purchased from Sigma. The TSK-G3000 SW $\times$ L column was from Toyo Soda (Tokyo, Japan); Fractogel EMD-TMAE 650 columns were obtained from Merck (Clevenot,

France); Mono P HR 5 × 20 columns and Polybuffer 74 were from Pharmacia (Uppsala, Sweden).

Cultivation method and yeast extracts

C. albicans strain 2091 was obtained from The Pasteur Institute (Paris, France). Nine clinical *Candida* isolates were used: three *C. albicans* isolates from urine, vagina and the mouth, and single isolates of *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii* and *C. pseudotropicalis*. All strains were grown for 48 h on Sabouraud's dextrose agar slants at 37°C.

The cells were harvested in 10 mM Tris-HCl buffer, pH 7.2, containing 150 mM NaCl (TBS), washed three times by centrifugation and suspended to a final concentration of 10⁹ cells/ml in the same buffer. The cells were disrupted for 10 min in a cell homogeniser MSK (B. Braun, Melsungen, Germany) with glass beads (0.45–0.55 mm) with cooling under CO₂.

Disrupted yeasts were centrifuged at 100 000 *g* for 30 min at 4°C, to remove glass beads, unbroken cells and cell debris. The supernatant fluid was taken as the cytoplasmic yeast extract and used for the assays or stored at –80°C until required. Protein concentration was determined as described by Bradford,¹³ with bovine serum albumin as a standard. All crude extracts used had the same protein concentration.

Enzyme purification

All the procedures were performed by high performance liquid chromatography (HPLC, Laboratoires Merck Clevenot, France).

For ion-exchange chromatography, 1 ml of yeast extract was applied to a Fractogel EMD-TMAE 650 column 150 × 10, previously equilibrated with 25 mM Tris-HCl, pH 7.4. A linear gradient was performed in the same buffer from 0 to 0.5 M NaCl in 30 min with a flow rate of 30 ml/h. Samples of each 0.5-ml fraction were tested with L-arginine-AMC (L-Arg-AMC).

For gel filtration, the active fractions from the ion-exchange column were pooled and concentrated by ultra-filtration with Centricon 10 (Amicon), and submitted to a TSK G3000 SW × L column 30 × 7.8, equilibrated with 25 mM phosphate buffer, pH 7.4. Elution was at 18 ml/h flow rate; usually, 0.5-ml fractions were collected and samples were assayed for enzymic activity.

Enzymic activity assays

The enzyme activity was monitored by a fluorogenic method with the AMC substrates. Ten µl of freshly prepared yeast extracts or of each fraction from the enzyme purification steps were incubated with 20 µM of fluorogenic substrate in 50 mM TBS, pH 7.2, to a final volume of 200 µl. After incubation for 15 min at 37°C, the reaction was stopped by adding 1 ml of ethanol. The fluorescence was measured at 440 nm with excitation at 380 nm for AMC.¹⁴ One U of L-Arg-

AMC activity was the amount catalysing hydrolysis and releasing 1.0 µmol of AMC/min.

The effect of pH on enzyme activity was studied with the purified enzyme and the following buffers: 50 mM sodium acetate, 50 mM Tris-HCl or 50 mM carbonate-bicarbonate with 0.15 M NaCl adjusted to the appropriate pH value from 4 to 10.

For protease inhibitor assays, 10 µl of enzyme solutions were pre-incubated at room temperature for 15 min with inhibitor before the addition of substrate. Controls were done under the same conditions but without protease inhibitors.

Determination of pI

For chromatofocusing, 1 ml of cytoplasmic yeast extract in starting buffer (25 mM imidazole-HCl, pH 6.6) was injected into a Mono P column HR 5 × 20 previously equilibrated with the same buffer. Proteins were eluted at a flow rate of 30 ml/h with a linear pH 6.6–2.7 gradient established with 30 ml of 1/8 polybuffer 74, pH 2.7. Each 1-ml fraction, neutralised with one volume of 250 mM Tris-HCl, pH 7.2, was assayed with L-Arg-AMC.

Polyacrylamide gel electrophoresis

Samples of purified enzyme fractions were dissolved in SDS 2%, β-mercapto-ethanol 2% solution, boiled for 5 min and analysed by SDS-PAGE as described by Laemmli.¹⁵ The acrylamide concentrations in stacking and separating gels were 3% and 10%, respectively. Gels were stained by the silver nitrate procedure according to Merrill *et al.*¹⁶

Visualisation of the proteolytic activity on polyacrylamide gel electrophoresis

AMC-substrate was added to the separating gel mixture to a concentration of 1 mg/ml before polymerisation. Pre-stained mol. wt markers were boiled and run on each gel as a guide to apparent molecular masses. However, samples containing enzyme were not heated. SDS-PAGE was performed at 4°C at 15 mA. To re-nature the enzyme, the gels were first rinsed briefly in de-ionised water and washed four times in Triton X-100 0.1% for 30 min at 4°C, then incubated at 37°C for 30 min in 50 mM TBS, pH 7.2. Enzyme activity was seen as fluorescent blue bands under long wave-length UV light.¹⁷

Detection of proteolytic activity in native gel electrophoresis

Samples of protein solution were mixed with an equal volume of sucrose and bromophenol blue and electrophoresed without prior heating in acrylamide 10% w/v/bisacrylamide 0.8% w/v gels.¹⁸

For enzyme detection, the gel was sliced into 2.5-mm horizontal strips. Each strip was then incubated at 37°C in 20 µM AMC-substrate in 50 mM TBS, pH 7.2,

for 1 h at 37°C. Absorbance was read at 440 nm with excitation at 380 nm and bands of peptidase activity were detected as absorbance peaks due to the production of AMC.

Results

With 7-AMC-derivatives, the cytosoluble extract of *C. albicans* was found to contain an active enzyme that had the properties of an aminopeptidase. This enzyme was purified by HPLC. Fig. 1 shows the protein activity profile for the purification of this aminopeptidase on a Fractogel EMD-TMAE 650, an anion-exchange chromatography column.

The active fraction from purification on the anion-exchange chromatography column was concentrated by centricon membrane and then applied to the gel filtration column TSK G3000 SW $\times$ L at pH 7.2. Activity monitored with L-Arg-AMC appeared as a sharp symmetrical peak (fig. 2). Thus the two chromatography columns allowed a rapid 20-fold total purification with high recovery of activity (table I). This purified peptidase had an optimal activity at pH 7.2 with L-Arg-AMC as substrate, similar to the crude enzyme extract (fig. 3).

Analysis of the purified protein by SDS-PAGE on 10% gels gave a single protein band with an M_r of 52 kDa (fig. 4, lane A). This result was confirmed by native polyacrylamide gel electrophoresis. The gels

were sliced and incubated with L-Arg-AMC substrate as described in *Materials and methods*. A single 52-kDa band that hydrolysed L-Arg-AMC was revealed (data not shown). Furthermore, analysis of the purified protein or the cytoplasmic extract gave a single blue band due to release of 7-AMC when they were electrophoresed in a polyacrylamide 10% gel containing L-Arg-AMC (fig. 4, lanes B and C). This result indicated an M_r of 52 kDa, which agreed with its elution in the void volume of the TSK G3000 SW $\times$ L column.

Determination of *pI*

Chromatofocusing on a Mono P column was used to determine the iso-electric point. When the *C. albicans* extract was subjected to a linear 6.6–2.7 pH gradient, the L-Arg-AMC peptidase was detected as a single peak of activity in the acidic fraction at *c.* pH 4.2 (fig. 5).

Inhibition sensitivity

The effects of various inhibitors on peptidase activity were determined (table II). *O*-Phenanthroline and EDTA, potent inhibitors of metallopeptidase, completely blocked enzyme activity. However, phosphoramidon—which is also a metallo-enzyme inhibitor—did not inhibit this activity at a concentration of 0.5 mM. Metals with a high affinity for

Fig. 1. Fractogel EMD-TMAE 650 ion-exchange chromatography of 100 000 g *C. albicans* extract. A 30-ml linear gradient of 25 mM Tris-HCl, pH 7.4, with 0–0.5 M NaCl was used for this purification step. The flow rate was 30 ml/h and samples of each 1.0-ml fraction were assayed for enzymic activity (■).

Fig. 2. After a first fractogel EMD-TMAE 650 ion-exchange chromatography, the pooled active fractions were injected into a gel filtration TSK G3000 SW $\times$ L column. The flow rate was 18 ml/h and samples of each fraction were assayed for activity with 20 μ M L-Arg-AMC as substrate (■). Calibration standards (arrows) were: lactate dehydrogenase, 145 kDa; ovotransferrin, 77 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; myoglobin, 17 kDa.

Table I. Purification of *C. albicans* metallopeptidase

Purification step	Volume (ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification factor	Recovery (%)
Cell extract	1	4.5	437.3	1967.8	...	100
Fractogel EMD-TMAE	0.5	0.46	2029.85	466.86	4.6	23.7
TSK G3000	0.5	0.025	15903.6	397.59	36.3	20.2

The purification started with 1 ml of cell-free extract (100 000 *g* supernate). The enzyme activity was determined at 37°C with L-Arg-AMC as substrate.

Fig. 3. Effect of pH on activity of *C. albicans* aminopeptidase. The maximal activity observed was set as 100% relative enzyme activity. Enzyme assays were performed with 20 μ M of L-Arg-AMC in buffers at different pH values as described in *Materials and methods*.

sulphydryl groups, such as Hg^{2+} and Zn^{2+} , inactivated the enzyme readily. Other sulphydryl reagents, such as iodoacetamide or *N*-ethylmaleimide, did not inactivate

the enzyme under the conditions used in these studies. Dithiothreitol, a reductive splitter of disulphide linkages, had a low inhibitory effect. Aminopeptidase activity was not inhibited by the serine protease inhibitor PMSF or by the chymotrypsin inhibitor TLCK.

Substrate specificity

In addition to L-Arg-AMC, the purified *C. albicans* 2091 metallopeptidase was able to cleave L-Leu-AMC. These substrates gave specific activities of 418 and 290 μ mol AMC released/min/mg of protein, respectively by spectrofluorometry (table III).

Aminopeptidase activity in other *Candida* species

Three *C. albicans* strains isolated from urine, vagina and mouth, and *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii* and *C. pseudotropicalis* were examined for the expression of aminopeptidase activity (table IV). All these yeasts

Fig. 4. SDS-PAGE analysis of the enzyme fraction after the two-step purification procedure described in figs. 1 and 2. The electrophoresis was done under reducing conditions. Numbers on the left are calibration standards: β -galactosidase, 116 kDa; pyruvate kinase, 58 kDa; fumarase, 48 kDa; lactic dehydrogenase, 36 kDa; lane A, purified enzyme stained by the silver procedure; B and C, blue AMC colour bands produced when the purified enzyme or crude extract, respectively, were electrophoresed in acrylamide gel containing L-Arg-AMC as substrate.

expressed a peptidase activity in their cytoplasmic extract that cleaved L-Arg-AMC with a pH optimum of 7.2. Furthermore, the peptidase activity in these species and in clinical isolates of *C. albicans* had the same inhibitor sensitivity as the *C. albicans* 2091 metallopeptidase. Therefore, extracts of all isolates tested contained an aminopeptidase that resembled the *C. albicans* 2091 enzyme.

Table II. Effects of various inhibitors on the purified *C. albicans* metallopeptidase activity at pH 7.2

Compound	Final concentration	Percentage inhibition
Control		0
CaCl ₂	1 mM	34
MnCl ₂	1 mM	5
HgCl ₂	1 mM	95
ZnCl ₂	1 mM	82
EDTA	1 mM	93
Iodoacetamide	1 mM	29
DTT	1 mM	38
<i>o</i> -Phenanthroline	1 mM	93
PMSF	1 mM	0
NEM	1 mM	0
Phosphoramidon	0.5 mM	0
TLCK	1 mM	23
E64	10 μ M	20

Discussion

In the present work, aminopeptidase activity was identified in *C. albicans* by a highly sensitive method with L-Arg and L-Leu 7-amino-4-methylcoumarin as substrates. The activity of enzyme in *C. albicans* cell lysates was 418 and 290 U/mg protein for L-Arg-AMC and L-Leu-AMC respectively. The purified peptidase showed optimum activity at pH 7.2 and an iso-electric point of 4.2, a K_m of 50 μ M and a V_{max} at 19 mM AMC released/min/mg of protein for L-Arg-AMC.

Anion-exchange chromatography and gel filtration of the *C. albicans* extract yielded a purification of 36-fold and indicated a single protein of 52 kDa. This result was confirmed by native SDS-PAGE or gel-electrophoresis with L-Arg-AMC as substrate co-

Fig. 5. Chromatofocusing on Mono P column of a 100000 g *C. albicans* extract in 25 mM imidazole buffer, pH 6.6. Elution formation of a pH 6.6–2.7 gradient with 45 ml of polybuffer/HCl, pH 2.7; flow rate: 30 ml/min. Fractions were monitored for protein content at 280 nm and 10- μ l samples of each fraction were assayed for activity with L-Arg-AMC, the hatched area corresponds to the active fraction.

Table III. Rates of hydrolysis of various 7-AMC substrates (20 μ M final concentration) by purified metallopeptidase of *C. albicans*

Substrate	Rate of hydrolysis (μ mol AMC released/min/mg of protein)
L-Arg-AMC	418
L-Leu-AMC	290
L-Lys-AMC	42.2
L-Tyr-AMC	15
D-Ala-Leu-Lys-AMC	No detectable activity
N-t-BOC- β -Benzyl-Asp-Pro-Arg-AMC	
N-Methoxysuccinyl-Ala-Ala-Pro-Val-AMC	
N-Succinyl-Leu-Leu-Val-Tyr-AMC	
Lys-Ala-AMC	
N-Succinyl-Ala-Ala-Ala-AMC	
N-Succinyl-Ala-Ala-Phe-AMC	
N-CBZ-Gly-Gly-Arg-AMC	
N-Succinyl-Gly-Pro-Leu-Gly-Pro-AMC	

Table IV. Rate of hydrolysis of L-Arg-AMC substrate (20 μ M final concentration) by cytoplasmic extract of various *C. albicans* isolates and other *Candida* spp.

Test strain	Rate of hydrolysis (μ mol AMC released/min/mg of protein)
<i>C. albicans</i> 2091	390
Isolate from vagina	205
Isolate from urine	328
Isolate from mouth	347
<i>C. glabrata</i>	84.2
<i>C. tropicalis</i>	106
<i>C. parapsilosis</i>	124
<i>C. krusei</i>	276
<i>C. guilliermondi</i>	330
<i>C. pseudotropicalis</i>	306

polymerised with acrylamide. These procedures enabled the M_r of the aminopeptidase to be determined. However, this enzyme failed to cleave gelatin co-polymerised in SDS-PAGE, in contrast with the two serine proteinases of *C. albicans* with M_r 50000 and M_r 60000.¹² This result shows that this enzyme has a narrow specificity for short peptides.

The lack of inhibition by NEM and iodoacetamide excluded the enzyme from the cysteine proteinase class (EC 3.4.22). PMSF had no effect on the activity, excluding it from the serine proteinase class (EC 3.4.21). However, since 1 mM *o*-phenanthroline and 1 mM EDTA completely blocked the enzyme activity, it is likely that this peptidase is a member of the metallo-enzyme class (EC 3.4.24). The optimum activity at pH 7.2 and the decreased activity with Zn^{2+} were in agreement with the known slightly alkaline pH optimum of metalloproteinases. Metalloproteinases contain zinc, and additional Zn^{2+} in the assay medium usually does not increase activity but may decrease it.¹⁹

Different proteases had been described in *C. albicans* with albumin, keratin or fluorogenic peptides as substrates. Among them, Rchel^{2,4} has purified an extracellular acid proteinase—first reported by Staib²⁰—with BSA as nitrogen source. This enzyme has a mol. wt of 42000, an iso-electric point of 4.5, an optimum activity at pH 4 and is inhibited by pepstatin and chymostatin.⁴ Several intracellular proteinases of *C. albicans* have been identified and characterised with peptide substrates.^{6,9,12,23} The properties of the present metallo-enzyme acting at neutral pH differ from those of the acid or neutral yeast proteinase described in *C. albicans*. This aminopeptidase differs from the metal ion-dependent aminopeptidases described by Logan *et al.*¹² and Portillo and Gancedo.²¹ These enzymes present a much higher mol. wt (243000 and 67000 respectively) than that reported here for the *C. albicans* enzyme.

Our data demonstrate the existence of an aminopeptidase in *C. albicans* and similar activity in other *Candida* spp. The occurrence of a peptidase activity in all *Candida* strains tested having a pH optimum of 7.2, similar inhibitor sensitivity and similar substrate specificity provides evidence that this peptidase could be a major enzyme in all *Candida* strains.

The biological function of the metallopeptidase described in this paper remains to be determined. Its optimal activity was at pH 7.2 and it will be of interest to determine if the present aminopeptidase is directly involved in the mechanism of tissue invasion by *C. albicans*. This enzyme could be a new target for the development of anti-fungal peptidyl drug strategy based on peptidase inhibitors.

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