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Original Scientific Paper

Aspartic Proteases from Basidiomycete *Clitocybe nebularis*

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Abstract. We have isolated aspartic proteases by affinity chromatography from wild growing basidiomycete *Clitocybe nebularis*. Pepstatin A sensitive fractions from size exclusion chromatography were subjected to Concanavalin A affinity chromatography. N-terminal sequences of the three bands resolved on SDS-PAGE showed sequence similarity to the A01.018 group of family A1 aspartic proteases of the MEROPS classification. The diversity of putative aspartic proteases found in *Clitocybe nebularis* basidiocarp extracts is considerable and shows the great potential of basidiomycetes as a source of unique proteases that could find use in biotechnological applications and drug design.

Keywords: aspartic protease, affinity chromatography, concanavalin A, pepstatin A, basidiomycete, *Clitocybe nebularis*

INTRODUCTION

Aspartic proteases (APs) (EC 3.4.23) are one of the four main groups of proteolytic enzymes that differ according to their functional groups at the active site. APs are endopeptidases that depend on one or two aspartic residues for their catalytic activity. They show maximal activity at acidic pH and are inhibited by pepstatin A. They are widely distributed among vertebrates, plants, nematodes, parasites, fungi, viruses and some bacteria and archaea.[1,2] APs have been used extensively in the dairy industry as milk-coagulating enzymes in the manufacture of cheese.[3] They are also important virulence factors and are involved in the pathogenicity of many viruses, including immunodeficiency viruses,[4] and in pathogenic fungi,[5] among which is *Candida albicans*, whose secreted aspartyl proteinases have been most studied.[6]

According to the MEROPS database,[7] aspartic proteases are now grouped, on the basis of amino acid sequence similarity, into 14 different families, which are assembled into six clans based on their evolutionary relationship and tertiary structure. Fungal APs are distributed among families A1, A2 and A11 of clan AA and family A22 of clan AD, the majority belonging to the A1 family, together with pepsin-like enzymes from many different origins. Although many APs have been described from ascomycetes, only a few have been described in basidiomycetes, including basidiomycetous yeasts *Cryptococcus neoformans*[8,9] and *Phaffia rhodozyma*[10] and three mushrooms: *Amanita muscaria*,[11] *Pleurotus ostreatus*[12] and *Polyporus tulipiferae* (formerly *Irpex lacteus*).[13] Apart from a *Cryptococcus neoformans* AP that belongs to the A22 family, all other APs described from basidiomycetes belong to the A1 family. Some APs for which no sequence data is available have been described from other basidiomycetes, and are not listed in the MEROPS database. These are a 25 kDa pepstatin A sensitive protease from *Phanerochaete chrysosporium*,[14] a 35 kDa pepstatin A sensitive enzyme with pronounced milk-clotting activity from *Russula decolorans*,[15] a 50 kDa pepstatin A sensitive aspartic proteinase from *Laetiporus sulphureus*[16] and a 36 kDa pepstatin A sensitive aspartyl protease form *Ustilago maydis*.[17]

For the past few years we have been engaged in characterization of the astonishing diversity of proteolytic activities in wild mushrooms.[18] In particular, we have focused on the endogenous proteolytic system of our model mushroom, *Clitocybe nebularis*, comprising proteases and their cognate inhibitors.[18,19] In the present study we describe the partial purification and characterization of proteolytic activities belonging to the aspartic protease family from basidiomycete *Clitocybe nebularis*.

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EXPERIMENTAL

Preparation of crude protein extract

Basidiocarps or fruiting bodies of basidiomycete *Clitocybe nebularis* (also classified as *Lepista nebularis*) were collected from their natural habitat (oak forest in Kras region, Slovenia) and frozen at −20 °C. After pressing the thawed basidiocarps through a sieve filter, crude protein extract was obtained by a three phase partitioning method using *t*-butanol and ammonium sulphate precipitation according to Dennison and Lovrien,[20] followed by dialysis of the precipitated protein against 0.1 mol dm^{-3} acetate buffer, pH 5.5, containing 0.3 mol dm^{-3} NaCl.

Size exclusion chromatography

Proteins were separated on preparative (4 × 110 cm, 42 ml/h flow rate with 14 ml fraction volume) and analytical (1.5 × 130 cm, 12 ml/h flow rate with 4 ml fraction volume) Sepharose S200 columns equilibrated with 0.1 mol dm^{-3} Na-acetate buffer, pH 5.5 containing 0.3 mol dm^{-3} NaCl.

Concanavalin A affinity chromatography

After size exclusion chromatography, fractions that showed enzymatic activity at pH 3.5 that was inhibited by pepstatin A were pooled and applied to an affinity column of Concanavalin A-Sepharose (Concanavalin A covalently bound to Sepharose 4B, Pharmacia Fine Chemicals). The column was then washed with 0.05 mol dm^{-3} Tris-HCl, 0.5 mol dm^{-3} NaCl, pH 7.5 and bound proteins eluted with the same buffer, but containing 0.6 mol dm^{-3} methyl-α-D-glucopyranoside (Sigma). Fractions showing proteolytic activity were pooled and applied to analytical size exclusion chromatography.

Protein concentration

Protein concentrations in solution were determined by absorbance at 280 nm, using a Perkin Elmer UV-Vis spectrophotometer λ18.

Enzyme activity assays

Proteolytic activities were followed during the isolation procedure using fluorescein thiocarbamoyl-haemoglobin (FTC-haemoglobin) as substrate in 0.1 mol dm^{-3} acetate buffer (pH 3.5) and in 0.1 mol dm^{-3} Tris-HCl buffers (pH 7.0 and pH 11.0). Soluble peptide products were determined fluorimetrically with excitation at 490 nm and emission at 525 nm ($F_{490/525}$), as described for fluorescein thiocarbamoyl-casein (FTC-casein).[21] The influence of different inhibitors on proteolytic activity was tested by incubating each inhibitor, at the indicated concentration, with the enzyme in assay buffer prior to the addition of substrate. The following inhibitors were used: 6 μmol dm^{-3} pepstatin A (Peptide Institute Inc.), 2 mmol dm^{-3} EDTA (Ethylenediamine tetraacetic acid, Serva), 4mmol dm^{-3} pefabloc SC (AEBSF; 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, Roche) and 100 μmol dm^{-3} chymostatin (Sigma).

Zymography

For analysis of proteolytic activity, Novex® Blue Casein zymogram pre-cast 4−16% gradient polyacrylamide gels (Invitrogen) were used according to the manufacturer's instructions. After washing in 2.5 % Triton-X 100 solution for 1 hour to remove SDS, zymograms were developed in developing solution (100 mmol dm^{-3} Na-acetate, 200 mmol dm^{-3} NaCl, 0.02 % (w/v) Brij 35) at pH 3.5 and room temperature for 20 h; proteolytic activity was visualized as clear bands against a blue background. Prestained SDS-PAGE standards of 21.4−113 kDa (Bio-Rad) were used for estimating apparent molecular mass.

Isoelectric focusing and SDS-PAGE

Isoelectric focusing was performed using the Phast System (Pharmacia-LKB), according to the manufacturer's instructions. Precast pH 3.0−9.0 gradient gels were calibrated with marker proteins with pI values ranging from 3.5 to 9.3 (Amersham Pharmacia Biotechnology). For SDS-PAGE, proteins were separated on 10 % polyacrylamide gels with 0.1 % SDS together with Low Molecular Weight (LMW) markers of 14.4–97.0 kDa (Amersham Pharmacia Biotechnology) using a mini-Protean II apparatus (Bio-Rad). Proteins were visualized by Coomassie Brilliant Blue R-250 staining according to the standard protocol or by the reversible, negative zinc-imidazole staining procedure as described.[22]

N-terminal sequence analysis

Automated amino acid sequencing was performed in a Procise liquid pulse sequencer (Applied Biosystems) connected on-line to a model 120 A analyzer. Proteins were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. Bands were stained with Coomassie Brilliant Blue R-250, excised and analyzed.

Sequence similarity analysis

Amino acid sequences were compared to N-terminal sequences of aspartic proteases listed in the MEROPS database.[7] Multiple sequence alignments were performed using the ClustalW algorithm in the BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

RESULTS AND DISCUSSION

Characterization of proteolytic activities from Clitocybe nebularis

Crude protein extract from *Clitocybe nebularis* basidiocarps was fractionated by preparative size exclusion

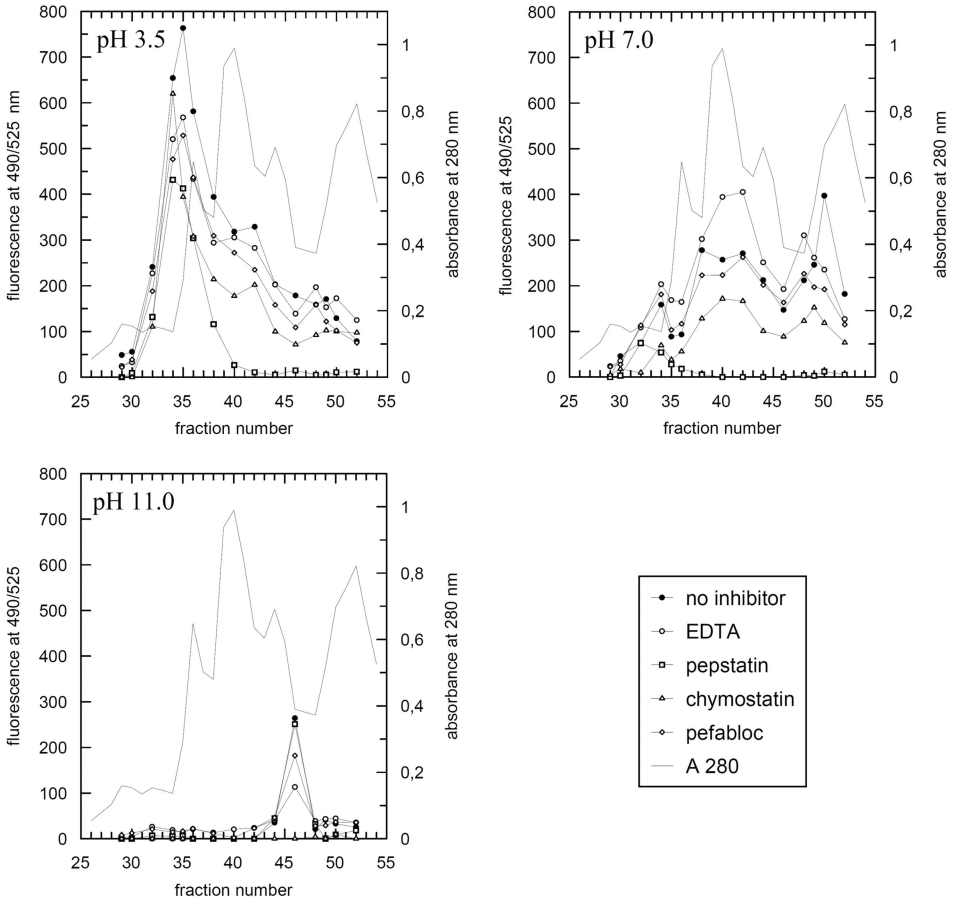

Figure 1. Size exclusion chromatography of crude protein extract from *C. nebularis* on preparative Sepharose S200 column. Proteolytic activities were measured against FTC-haemoglobin at pH 3.5, pH 7.0 and pH 11.0, in the absence and presence of class specific inhibitors. Symbols: absorbance at 280 nm (—); proteolytic activity is expressed as $F_{490/525}$ with no inhibitor added (●), and in the presence of EDTA (○), pepstatin A (□), chymostatin (△), or pefabloc SC (◇).

chromatography and proteolytic activity in fractions measured at pH 3.5, pH 7.0 and pH 11.0 (Figure 1). The highest proteolytic activity was observed at acidic pH, mostly in high molecular mass proteins. Activities measured at neutral pH were more evenly distributed over the entire molecular mass range. Only low proteolytic activity was detected at alkaline pH. Class specific protease inhibitors (pepstatin A, EDTA, chymostatin and pefabloc) were used to identify the type of proteolytic activity (Figure 1). The most effective inhibitor at both pH 3.5 and 7.0 was pepstatin A, indicating the presence of aspartic proteases in the mid and low molecular mass proteins. Although most pepsin-like aspartic proteases have an acidic pH optimum, some also exhibit activity under neutral conditions,[23] such as PEP2 aspartic proteinase from *Aspergillus fumigatus*[24] and aspartic proteinases from *Botrytis cinerea*.[25] At pH 7.0, proteolytic activity was evenly spread over proteins in the mid molecular mass region and was partially inhibited by chymostatin. No inhibition by EDTA was observed, rather, EDTA stimulated proteinase activity at pH 7.0. A similar phenomenon has been observed for aspartic proteinase activity from *Botrytis cinerea*[25] and is probably due to destabilization of the haem group and globin association by the chelator, thereby making the haemoglobin substrate more accessible to proteolysis.[25] On the other hand, at pH 11.0 the low molecular mass proteolytically active fractions were completely inhibited by chymostatin, while pefabloc and EDTA showed incomplete inhibition and no inhibition was exhibited by pepstatin A.

Concanavalin A affinity chromatography

Fractions 40 to 45 from preparative size exclusion chromatography that showed complete inhibition by pepstatin A were pooled and separated by Concanavalin A (ConA) affinity chromatography. Proteolytically active fractions eluted by methyl-α-D-glucopyranoside were pooled (ConA peak) and analyzed by SDS-PAGE (Figure 2) and casein zymography (Figure 3B, lane A). Three prominent protein bands were observed on denaturing SDS-PAGE and named, according to their relative molecular masses, ConA58, ConA40 and ConA32. On casein zymography only two active bands were

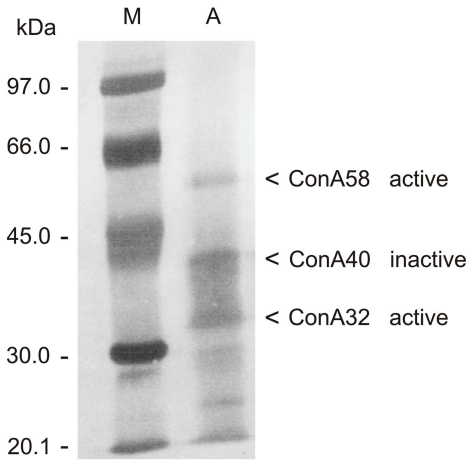

Figure 2. SDS-PAGE analysis of pooled proteolytically active fractions with affinity for ConA. Fractions were eluted with methyl-α-D-glucopyranoside from ConA affinity chromatography (*lane A*). Proteins were visualized by Coomassie Blue staining. *Lane M*, protein molecular weight markers. The proteolytic activity of individual bands was determined by casein zymography.

observed. In order to relate SDS-PAGE and zymography-obtained bands, given that molecular masses determined by zymogram analysis are not reliable,[18,26] we performed SDS-PAGE of the ConA peak under mildly denaturing conditions. Bands were visualized by reversible, negative zinc-imidazole staining, excised and subjected to zymogram analysis. The results confirmed that ConA58 and ConA32 bands were proteolytically active, while ConA40 band showed no activity under these mildly denaturing conditions (not shown). Molecular masses reported for APs isolated from basidiomycetes range from 25 kDa (*Phanerochaete chrysosporium* AP),[14] to 35 kDa (*Irpex lacteus* and *Russula decolorans* APs),[13,15] 36 kDa (*Phaffia rhodozyma* and *Ustilago maydis* APs),[10,17] 45 kDa (*Amanita muscaria* AP),[11] and 50 kDa (*Cryptococcus neoformans* CnAP1).[9]

The proteins of the ConA peak were separated on an analytical size exclusion column with high resolution characteristics. The elution profile comprised three proteolytically active peaks (Figure 3A), with the highest FTC-haemoglobin degrading activity concentrated in peak 3. Casein zymography of the three peaks, on the other hand, showed only two proteolytically active bands (Figure 3B). The first two peaks showed enriched proteolytic activity with an apparent molecular mass of 70 kDa, and peak 3 was enriched in proteolytic activity at an apparent molecular mass of 32 kDa. The major protein band of peak 3 had a pI of 5.3 (Figure 3C). The isoelectric point is in the range of those reported for other fungal APs, pI 5.5 for *Ustilago maydis* AP,[17] 4.9 for *Russula decolorans* AP,[15] 7.3 for *Phaffia rhodozyma* AP,[10] 4.5 for AP from *Trichoderma harzianum*,[27] and

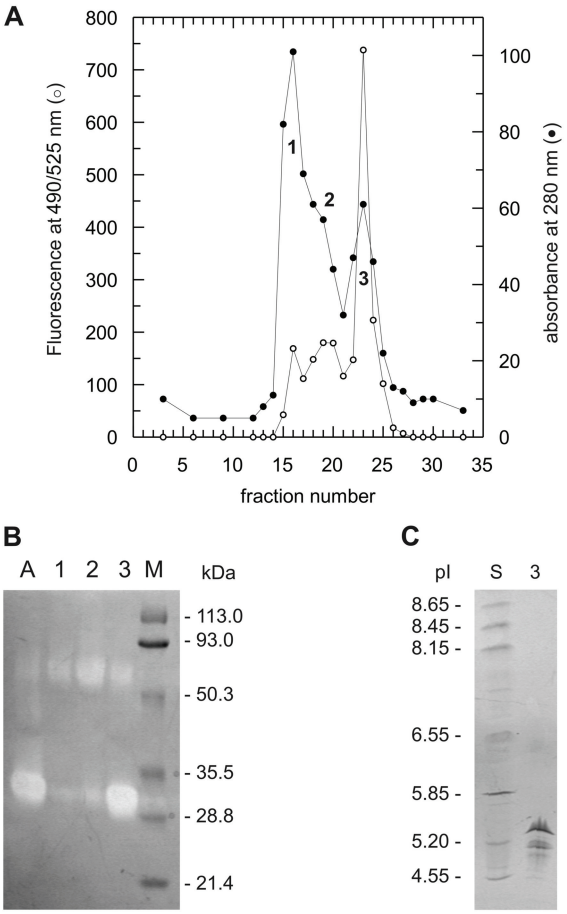

Figure 3. Characterization of proteolytically active fractions following ConA affinity chromatography. (**A**) Size exclusion chromatography on analytical Sepharose S200 column, absorbance at 280 nm (●) and proteolytic activity towards FTC-haemoglobin at pH 3.5 expressed as $F_{490/525}$ (○). Peak numbers correspond to lanes in panels B and C. (**B**) Casein zymography shows proteolytic activities as white bands on a dark background, *lane A*, pooled proteolytically active fractions from ConA which were applied to analytical size exclusion chromatography; *lanes 1, 2,* and *3* correspond to size exclusion peaks in panel A, *lane M*, protein molecular mass markers. (**C**) IEF analysis of size exclusion peak 3 in panel A, *lane S*, standard protein IEF markers.

3.5 for CnAP1 from *Cryptococcus neoformans*.[9]

Finally, N-terminal sequences of the three proteins eluted from Concanavalin A affinity column, which were resolved on SDS-PAGE (Figure 2), were determined. All three show a high percentage of sequence similarity to N-terminal sequences of aspartic proteases (Figure 4). The closest similarity was found to the A01.018 group of family A1 aspartic proteases of the MEROPS classification, with saccharopepsin as holotype (Figure 4). Members of the A01.018 group of family A1 are pepstatin A sensitive peptidases with two to

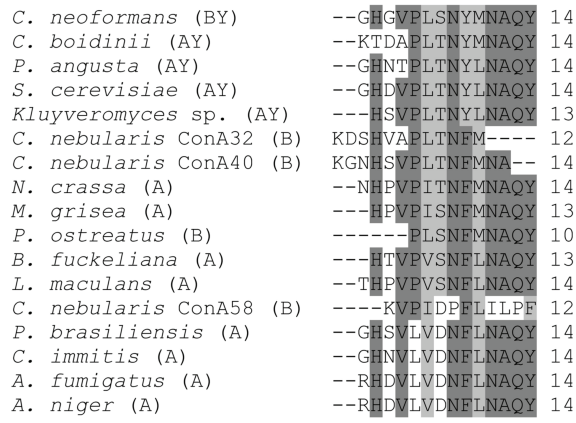

Figure 4. Alignment of N-terminal sequences of active (ConA58, ConA32) and putative (ConA40) *C. nebularis* aspartic proteases (CnebAPs) with selected APs of A01.018 group of family A1 aspartic proteases of the MEROPS classification ordered to give optimal alignment. The taxonomic group is given in parenthesis: B, basidiomycete, A, ascomycete and species growing as yeasts are marked with Y. Identical residues in at least 12 of 17 sequences (70 %) are highlighted in *dark gray* and similar residues in *light gray*; introduced gaps are shown as *hyphens*. MEROPS accession numbers of the sequences: *Cryptococcus neoformans* MER052647, *Candida boidinii* MER017039, *Pichia angusta* MER003036, *Saccharomyces cerevisiae* MER000941, *Kluyveromyces* sp. MER053817, *Neurospora crassa* MER001951, *Magnaporthe grisea* MER048480, *Pleurotus ostreatus* MER019987, *Botryotinia fuckeliana* MER054323, *Leptosphaeria maculans* MER020005, *Paracoccidioides brasiliensis* MER001469, *Coccidioides immitis* MER011983, *Aspergillus fumigatus* MER004337, *Aspergillus niger* MER001992.

five putative N-glycosylation sites and molecular masses that range from 39 kDa (*Aspergillus fumigatus*)[24] to 45 kDa (*Coccidioides immitis* aspartyl protease, *S. cerevisiae* protease A).[28,29] N-terminal sequences of ConA40 and ConA32 show 92 % and 70 % sequence similarity, respectively, to the N-terminal sequence of *Neurospora crassa* neurosporapepsin (A1) with a molecular mass of 42.9 kDa and subject to inhibition by pepstatin A.[30] The N-terminal sequence of ConA58 shows sequence similarity (50 %) to the N-terminal sequences of A1 family *Leptosphaeria maculans* and *Paracoccidioides brasiliensis* aspartic proteases. Surprisingly, within the A01.018 group the N-terminal sequence similarity of *Clitocybe nebularis* aspartic proteases, abbreviated as CnebAP (not to be confused with CnAP1 from *Cryptococcus neoformans*[9]), is not greater to basidiomycete aspartic proteases than to their ascomycete counterparts.

Clitocybe nebularis aspartic proteases (CnebAPs)

The proteolytic activities from basidiocarps of basidiomycete *Clitocybe nebularis*, have been characterized as aspartic type, on the basis of the isolation procedure, activities, inhibition specificity and N-terminal sequence similarity. In an alternative isolation procedure for aspartic type proteases, crude protein extract from *Clitocybe nebularis* basidiocarps was applied to pepstatin A affinity chromatography (not shown). The proteolytic activity was eluted in a single peak, with four dominant bands on SDS-PAGE ranging from 21 kDa to 55 kDa and two proteolytically active bands in casein zymography. We were unable to unequivocally assign these activities to the particular protein bands resolved on SDS-PAGE. N-terminal sequences were determined, however, for the 55 kDa protein band (VTITPNLANGDEVIT) and for the 48 kDa protein band (TTGTPINHNGDVNKKLGIFN), both showing sequence similarity with the unassigned AP from *Amanita muscaria* (MER015087).

Surprisingly, the N-terminal sequences of proteins isolated by pepstatin A affinity chromatography were additional to those isolated by ConA affinity chromatography. The fact that proteases obtained by pepstatin A affinity chromatography did not bind to ConA Sepharose may be explained either by lack of recognition by ConA lectin due to unconventional site or the type of glycosylation. In addition, by definition aspartic proteases should be specifically and reversibly inhibited by pepstatin A. The inability to attach proteases showing affinity for ConA Sepharose may be explained by the inaccessibility of their active site to pepstatin A bound to agarose.

Furthermore, not all protein bands obtained by affinity chromatography and bearing N-termini showing similarity to aspartic proteases showed activity on zymograms. The explanation may be that their activity had been lost during isolation, or that not all proteases have the ability to renature after removal of SDS. The lack of visible results of proteolysis can be attributed also to unsuitable substrate and choice of assay pH together with the possibility of the presence of non-peptidase homologues.

The extent of sequence similarity of all the N-terminal sequences obtained in this study to those of APs from other fungi was unexpected. AP molecules are bilobed proteins with the catalytic site in the form of a cleft located between the two homologous lobes, and two aspartic acid residues form the catalytic dyad. In the present-day enzymes, only very limited similarities in the amino acid sequences of the two lobes remain, but the three-dimensional structures are very similar.[23] Only limited sequence similarity at the N-terminal region was expected for APs from *C. nebularis*, since homology in this part of molecule is generally low. *Phaffia rhodozyma* aspartic protease shows 30−35 % sequence identity to other known APs, with major sequence elements characteristic of aspartic proteases conserved.[10]

Similarly, *Irpex lacteus* (or *Polyporus tulipiferae*) aspartic proteinase (ILAP) showed limited overall similarity to the aspartic proteinase family, and strict conservation of the essential residues around the two active-site aspartic acid residues.[13,31]

CONCLUSION

Basidiocarps of basidiomycete *Clitocybe nebularis* contain several proteolytic activities. Among them are several putative aspartic proteases, with different relative molecular masses and N-termini that have been isolated and partially characterized. ConA affinity chromatography of pepstatin A sensitive fractions from size exclusion chromatography revealed APs with N-terminal sequence similar to the saccharopepsin group of A1 family in the MEROPS database. The diversity of aspartic proteases found in *C. nebularis* basidiocarp extract is considerable and points to the great potential of the fungal proteolytic system as a possible source of unique proteases for biotechnological applications and drug design.

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SAŽETAK

Aspartatske proteaze iz basidiomiceta *Clitocybe nebularis*

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Afinitetnom kromatografijom izolirali smo aspartatske proteaze iz divlje rastućih basidiomiceta *Clitocybe nebularis*. Osjetljive frakcije pepstatina A dobivene kromatografijom isključenja po veličini (engl. *size exclusion chromatography*) bile su podvrgnute afinitetnoj kromatografiji s concanavalinom A. N-terminalne sekvence za tri vrpce dobivene na SDS-PAGE-u su pokazale sekvencijsku sličnost grupi A01.018 koja pripada familiji A1 aspartatskih proteaza prema MEROPS-ovoj klasifikaciji. Različitost pretpostavljenih aspartatskih proteaza nađenih u ekstraktima *Clitocybe nebularis* basidiocarp-a je značajna i daje veliki potencijal basidiomicetima kao izvoru jedinstvenih proteaza koje mogu poslužiti za biotehnološke primjene i u dizajnu lijekova.