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PRODUCTION OF EXTRACELLULAR PROTEASES BY HUMAN PATHOGENIC *TRICHODERMA LONGIBRACHIATUM* STRAINS*

L. KREDICS[1], ZSUZSANNA ANTAL[1], A. SZEKERES[2], L. MANCZINGER[2], ILONA DÓCZI[3], F. KEVEI[2] AND ELISABETH NAGY[1, 3]

[1]Hungarian Academy of Sciences and University of Szeged, Microbiological Research Group, P.O. Box 533, H-6701 Szeged, Hungary, [2]Department of Microbiology, Faculty of Sciences, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary, [3]Department of Clinical Microbiology, Faculty of Medicine, University of Szeged, Somogyi Béla tér 1, H-6725 Szeged, Hungary

*Dedicated to the 100th anniversary of the birth of Professor György Ivánovics

Running title: PROTEASES OF PATHOGENIC *TRICHODERMA* STRAINS

Keywords: *Trichoderma*, opportunistic fungi, virulence factors, extracellular proteases, trypsin, chymotrypsin

Abstract

Species belonging to the filamentous fungal genus *Trichoderma* are well known as potential candidates for the biological control of plant pathogenic fungi and as cellulase producers of biotechnological importance. Several data were published in the last decade also about the clinical importance of this genus, indicating that *Trichoderma* strains may be potential opportunistic pathogens of immunocompromised patients. However, there is a lack of information about the potential virulence factors of clinical *Trichoderma* strains. This study was designed to examine the extracellular proteolytic enzymes of six clinical *T. longibrachiatum* isolates.

Supernatants from induced liquid cultures of the examined strains were screened for proteolytic enzyme activities with 11 different chromogenic *p*-nitroaniline substrates. The production of trypsin-like, chymotrypsin-like and chymoelastase-like protease activities cleaving *N*-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide, *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, and *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide, respectively, was common among the examined strains. Separation of trypsin- and chymotrypsin-like activities by column chromatography revealed, that both systems are complex consisting of several isoenzymes. The pH-dependence of these two protease systems was also studied. Based on the results, the different isoenzymes seem to have different optimal pH values.

Extracellular proteolytic enzymes may be involved in the pathogenesis of *Trichoderma* strains as facultative human pathogens.

Introduction

Trichoderma species are imperfect filamentous fungi with multiple practical importance. Some *Trichoderma* species are very good cellulase producers therefore they are important for the biotechnological industry [1]. Certain *Trichoderma* strains are potential candidates for the biological control of plant pathogenic fungi, thanks to different mechanisms, e.g. antibiosis, competition and mycoparasitism [2].

Members of the genus *Trichoderma* are also on the growing list of facultative human pathogenic fungi causing infections in immunocompromised patients. A recent, detailed review is available about the clinical importance of the genus, covering 40 known cases with the involvement of *Trichoderma* strains [3]. Numerous cases of peritonitis have been reported in peritoneal dialysis patients, the causative agent was isolated from the peritoneal fluid. The number of cases of *Trichoderma* infections in transplant recipients is also significant, the fungus was recovered from different organs, e.g. skin, lung, liver, intestinal wall, sinuses and brain. The number of cases is growing from year to year, which is due to the expanding of the population of immunocompromised hosts. Further *Trichoderma* infections were documented since the preparation of the cited review: *T. longibrachiatum* strains were isolated from the subcapsular hepatic collection of a liver transplant recipient and from bronchoalveolar lavage and pleural drains of a pulmonary transplant patient [4] and from fatal necrotizing stomatitis of a neutropenic patient with malignant lymphoma [5]. A further case of fatal peritonitis due

to *Trichoderma* sp. was also reported in a patient undergoing continuous ambulatory peritoneal dialysis [6].

The most frequent etiologic agent within the genus is *T. longibrachiatum*; strains belonging to this species were involved in most of the known cases. Infections caused by members of other species of the genus, e.g. *T. harzianum* [7], *T. viride* [8], *T. koningii* [9], *T. pseudokoningii* [10] and *T. citrinoviride* [11] have also been reported. However, ITS sequence data revealed, that the reidentification of certain clinical strains – a *T. pseudokoningii* ([11], GenBank accession number Z82902) as well as a *T. koningii* and a *T. citrinoviride* (Kredics *et al.*, unpublished data, GenBank accession numbers AY328034 and AY328038, respectively) – as *T. longibrachiatum* seems to be necessary. Therefore it is conceivable, that a series of *Trichoderma* strains reported from other species have been misidentified, and the agents with a potential to cause diseases in immunocompromised patients are restricted almost exclusively to species *T. longibrachiatum*. An important taxonomic marker of this species is thermotolerant growth [12], which seems to be one of the most important properties for virulence. There is a lack of information about other potential virulence factors, e.g. the ability of growth at neutral pH, the utilisation of basic amino acids as carbon- and nitrogen source, the ability for hemolysis, the hydrophobicity of conidia, or the production of pigments, mycotoxins and proteolytic enzymes.

Data are available in the literature about the production of proteases by *Trichoderma* strains and about the role of these types of enzyme activities in the degradation of fungal [13] and bacterial [14] cells. We suppose, that extracellular proteases are among the potential virulence factors of *Trichoderma* strains as opportunistic pathogens, therefore we designed this study to examine the proteolytic abilities of six *T. longibrachiatum* strains isolated from clinical samples.

Materials and methods

Strains and culture conditions

Six clinical *T. longibrachiatum* isolates were involved in the experiments: strains UAMH 7955 from acute invasive sinusitis of a liver and small bowel transplant recipient [15], UAMH 7956 from lung, liver and intestinal wall of a bone marrow transplant recipient [16] and UAMH 9515 isolated from the peritoneal effluent of a female were purchased from the University of Alberta Microfungus Collection and Herbarium. Strains ATCC 201044 from a skin lesion of a pediatric patient with aplastic anemia [17] and ATCC 208859 from an HIV+ patient [11] derived from the American Type Culture Collection. *T. longibrachiatum* CBS 446.95 isolated from the lung of a man who died was from Centraalbureau voor Schimmelcultures.

For the induction of extracellular proteases, 10 g l^{-1} skim milk powder was incorporated as inducer into 30 ml liquid media (5 g l^{-1} KH_2PO_4, 1 g l^{-1} $NaNO_3$, 1 g l^{-1} $MgSO_4 \cdot 7H_2O$). After inoculation with conidial suspensions to a final concentration of 10^5 conidia ml^{-1}, the incubation was carried out in 50-ml Erlenmeyer flasks on a shaker at 200 rpm and 25°C. After 4 days of incubation the mycelial pellets were removed by centrifugation and enzyme activities were measured in the supernatants. Based on our experience about the

susceptibility and accuracy of measurements using *p*-nitroaniline substrates, the measured enzyme activities were considered significant, if the OD_{405} values were above 0.110.

Screening for extracellular proteases

The clinical *T. longibrachiatum* isolates were screened for extracellular protease activities with a series of chromogenic *p*-nitroaniline substrates derived from Sigma: L-Leu-*p*-nitroanilide, *N*-Acetyl-L-Leu-*p*-nitroanilide, *N*-Succinyl-L-Phe-*p*-nitroanilide, *N*-Benzoyl-L-Tyr-*p*-nitroanilide, *N*-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide, *N*α-Benzoyl-DL-Arg-*p*-nitroanilide, *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, *N*-Succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide, *N*-CBZ-L-Gly-L-Gly-L-Leu-*p*-nitroanilide, *N*-CBZ-L-Ala-L-Ala-L-Leu-*p*-nitroanilide and *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide. The substrates were dissolved in distilled water at a concentration of 1 mg ml^{-1}. One hundred µl of each supernatant was mixed with 100 µl substrate solution, resulting in reaction mixtures with substrate end concentrations of 500 µg ml^{-1}. Control mixtures were prepared for each strain from 100 µl culture supernatant and 100 µl distilled water. After incubation at 25°C for 1 hour, the optical densities of the samples were determined with a Labsystems Uniskan II microtiter plate spectrophotometer at a wavelength of 405 nm.

Gel filtration chromatography

The supernatants of induced cultures were concentrated tenfold by lyophilization and amounts of 2 ml were separated by column chromatography performed on a Sephadex G-100 gel bed (Pharmacia) of 0.9×60 cm dimensions. The eluent contained 1 g l^{-1} NaCl and 1 g l^{-1} $MgCl_2$. Trypsin- and chymotrypsin-like protease activities were measured in the fractions with *N*-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide and *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, respectively as described above, the end concentration of the substrates in the reaction mixtures was 100 µg ml^{-1}. Chromatographic profiles were prepared with the software MicroCal Origin 2.94. Molecular weights of the peaks were estimated using a calibration curve for globular peptides and proteins.

Studies on the pH dependence of trypsin-like and chymotrypsin-like protease activities

The substrates *N*-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide and *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide were dissolved in double concentrated McIlvain buffer solutions (mixtures of 0.2 M Na_2HPO_4 and 0.1 M citric acid in different proportions). One hundred µl of each supernatant was mixed with 100 µl substrate solution resulting in reaction mixtures with substrate end concentrations of 100 µg ml^{-1} and pH values between 2.0 and pH 9.0. Incubation and enzyme activity measurements were carried out as described above. Data were expressed as relative enzyme activities in the percentage of the maximal activity measured. pH-dependence profiles were prepared with Microsoft Excel 2002.

Results

Extracellular proteolytic activities produced by human pathogenic T. longibrachiatum *isolates*

Table I shows the protease activities produced by the six examined *T. longibrachiatum* strains measured with 11 different *p*-nitroaniline derivatives. The best producers of extracellular proteolytic enzymes proved to be strains UAMH 7956 and ATCC 208859 with culture supernatants cleaving 8 of the examined protease substrates. Variability was detected among the strains in the presence or absence of activities for most of the substrates; however, all of the strains produced trypsin-like, chymotrypsin-like and chymoelastase-like protease activities cleaving *N*-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide, *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide and *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide, respectively. Trypsin- and chymotrypsin-like activities were subjected to further analysis.

Column chromatographic profiles of trypsin-like and chymotrypsin-like protease activities

Fig. 1 shows the trypsin-like protease activity profiles of the examined strains measured from fractions after Sephadex G-100 column chromatography of crude supernatants derived from induced cultures. The profiles proved to be complex, suggesting the presence of several isoenzymes, and variability was detected among the strains. Three main peaks could be distinguished near the fractions 25, 35 and 55 (approximately 58, 29 and 6.4 kDa, respectively) in the case of strains ATCC 208859 and CBS 446.95. For the other strains only the first two peaks (UAMH 7955, UAMH 7956 and ATCC 201044) or just the first peak (UAMH 9515) was present. Fig. 2 shows the chymotrypsin-like protease activity profiles, which proved to be similar for all examined strains with one main peak between fractions 20 and 50 corresponding to a molecular weight range between 9.2 and 90 kDa. It is conceivable, that this peak is the result of the addition of the activities of more isoenzymes with similar

molecular weights. This is supported by the data about the pH-dependence of chymotrypsin-like activities.

pH dependence of trypsin-like and chymotrypsin-like protease activities

Our data about the pH-dependence of extracellular protease activities are shown in Fig. 3. The profiles do not follow the classic Gaussian optimum curve. Relatively high activity levels were detected in the case of the examined strains between pH 5 and 9 for both trypsin-like (Fig. 3a) and chymotrypsin-like (Fig. 3b) activities, suggesting that both types of activities consist of more isoenzymes, and the different isoenzymes seem to have different pH characteristics.

Discussion

Six human pathogenic *T. longibrachiatum* isolates were screened for the production of different types of extracellular proteases. All of the examined strains were able to produce certain chymoelastase-like, trypsin-like and chymotrypsin-like activities. The latter two types of protease activities were separated by the means of column chromatography, which revealed complex isoenzyme profiles suggesting that both systems consist of several isoenzymes. Based on the results of the studies on the pH-dependence of trypsin- and chymotrypsin-like protease activities, the different isoenzymes seem to have different pH optima between pH 5 and 9.

According to our previous results, protease production of clinical *Trichoderma* isolates depend on the culturing conditions in many cases: the activities of certain proteases proved to be higher in the supernatants of induced cultures shaken at 37°C than of those shaken at 25°C

[18]. Proteases with such characteristics may represent important virulence factors of opportunistic *Trichoderma* strains.

The cellulolytic, xylanolytic and chitinolytic systems of *Trichoderma* strains are well characterised, while the proteolytic enzyme system is relatively unknown in the case of this genus. Extracellular proteases are supposed to be of great importance for the biocontrol abilities of *Trichoderma* strains. The role of *T. harzianum* proteases in the biocontrol of *B. cinerea* was studied by Elad and Kapat [19]. Several acidic, neutral and basic extracellular proteases have been detected in the case of *T. harzianum* [20]. Geremia *et al.* isolated and cloned the gene *prb*1 encoding a basic protease related to mycoparasitism [21], while Flores et al. have demonstrated, that the over-expression of this gene resulted in improved biocontrol activity, suggesting the importance of proteases in the degradation of the protein components of the host cell wall and in the lysis of whole host cells [12]. An aspartyl protease of *T. harzianum* was also cloned and characterized [22].

Specific trypsin- [23, 24, 25] and chymotrypsin-like activities [24, 25] were also detected in the case of saprophytic *Trichoderma* strains with biocontrol potential, and Pra1, a trypsin-like protease of *T. harzianum* was purified and characterized [23]. Szekeres et al. isolated *p*-fluorophenylalanine resistant and morphological mutants of *T. harzianum* [26], several mutants were overproducers of trypsin- and chymotrypsin-like proteases and possessed better *in vitro* antagonistic properties against plant pathogenic fungi, than the wild-type strain. Studies are available about the effects of different environmental factors, e.g. low temperature [27], low water potential [28] and the presence of metal ions [29] on the *in vitro* activities of these protease systems. Data indicate in all of these cases, that the extracellular proteases of *Trichoderma* strains can remain active even under environmental conditions, which are already unfavourable for mycelial growth. Proteases seem to have great importance in the degradation of bacterial cells as well. In the case of a *T. harzianum* strain, the

supernatant derived from cultures induced by heat-inactivated *Bacillus subtilis* cells was fractionated on a Sephadex G-150 column and the detected enzyme profiles proved to be complex with at least 3 trypsin-like (approx. 5, 13 and 19 kDa in size) and 6 chymotrypsin-like proteases (between 12 and 43 kDa) [14].

In conclusion, it seems to be obvious, that *Trichoderma* strains evolved the ability of extracellular protease production to increase their survival advantage as competitive saprophytic and mycoparasitic organisms. However, this property may turn to a virulence factor, if these fungi get in touch accidentally with immunocompromised humans. Evidences are available for the possible involvement of proteolytic enzymes in aspergillosis, coccidioidomycosis and sporotrichosis [30], therefore we suppose that the production of extracellular proteases may contribute to the pathogenesis in the cases of opportunistic *Trichoderma* infections as well.

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Addresses of the authors:

Corresponding author: Dr. László Kredics, Hungarian Academy of Sciences and University of Szeged, Microbiological Research Group, P.O. Box 533, H-6701 Szeged, Hungary, Fax: 06 62 544 823, E-mail: kredics@bio.u-szeged.hu

Dr. Zsuzsanna Antal: same address as above

András Szekeres, Dr. László Manczinger, Dr. Ferenc Kevei: Department of Microbiology, Faculty of Sciences, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary. Fax: 06 62 544 823

Prof. Elisabeth Nagy, Ilona Dóczi: Department of Clinical Microbiology, Faculty of Medicine, University of Szeged, Somogyi Béla tér 1, H-6725 Szeged, Hungary

Figure legend:

Fig. 1. Trypsin-like protease enzyme activity profiles of human pathogenic *T. longibrachiatum* strains (separated by Sephadex G 100 column chromatography).

Fig. 2. Chymotrypsin-like protease enzyme activity profiles of human pathogenic *T. longibrachiatum* strains (separated by Sephadex G 100 column chromatography).

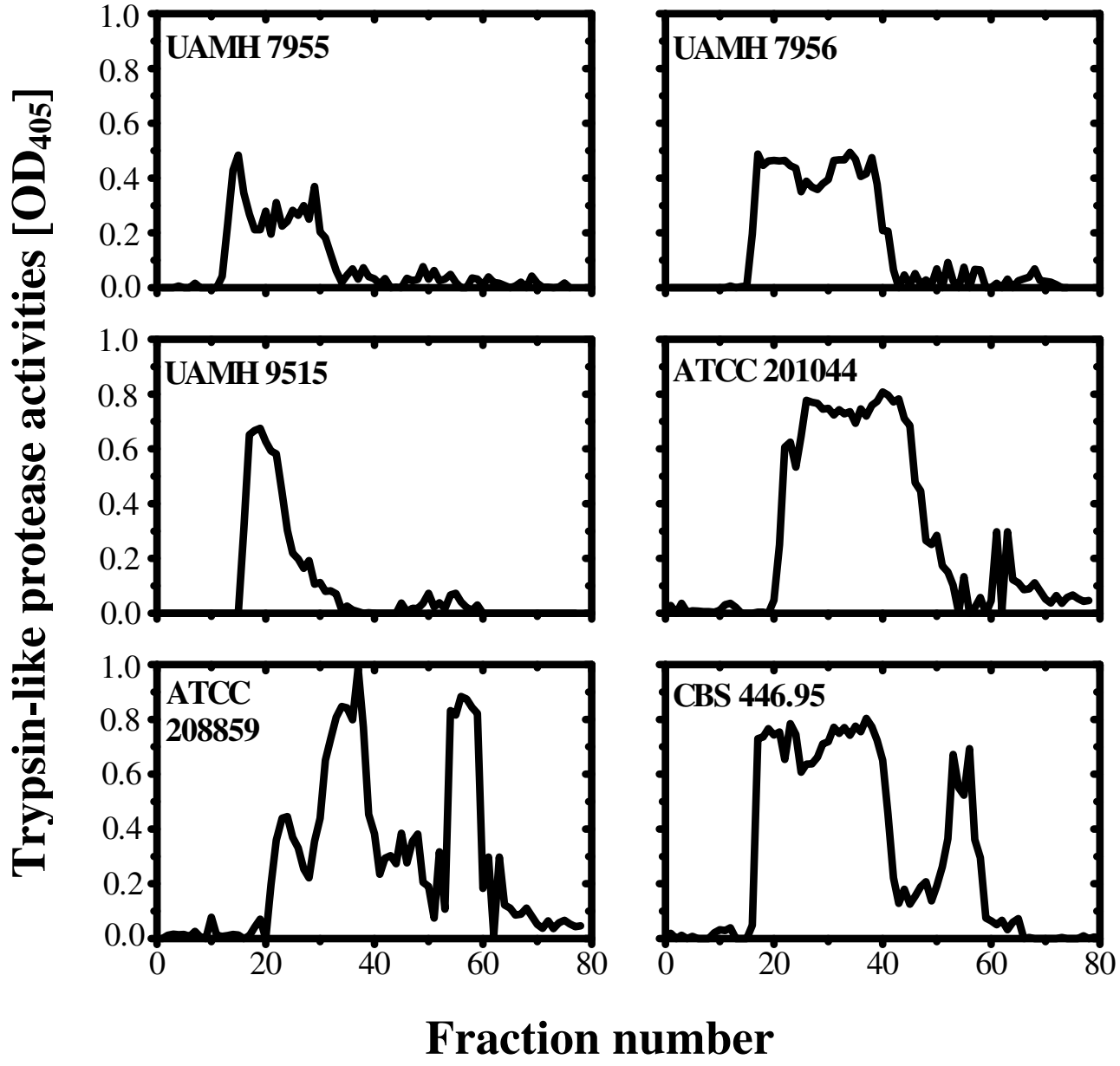
Fig. 3. pH-dependence of trypsin-like (a) and chymotrypsin-like (b) protease activities of human pathogenic *T. longibrachiatum* strains. ■: UAMH 7955, ■: UAMH 7956, ■: UAMH 9515, ■: ATCC 201044, ■: ATCC 208859, □: CBS 446.95

Table I

Proteolytic enzyme activities of human pathogenic T. longibrachiatum *strains measured with different* p-*nitroanilide substrates*

Substrate	*T. longibrachiatum* strains					
	UAMH 7955	UAMH 7956	UAMH 9515	ATCC 201044	ATCC 208859	CBS 446.95
L-Leu-*p*-nitroanilide	0.035[a]	**0.155**	0.036	0.071	0.049	**0.125**
N-Acetyl-L-Leu-*p*-nitroanilide	0.008	**0.185**	0.040	0.056	0.070	0.022
N-Succinyl-L-Phe-*p*-nitroanilide	0.054	**0.227**	0.029	**0.148**	**0.171**	0.097
N-Benzoyl-L-Tyr-*p*-nitroanilide	0.000	0.022	**0.117**	0.000	0.068	0.015
N-Benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide	**0.196**	**0.171**	**0.782**	**0.649**	**0.780**	**0.499**
*N*α-Benzoyl-DL-Arg-*p*-nitroanilide	0.000	0.081	0.019	0.000	**0.123**	0.104
N-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide	**1.354**	**1.702**	**0.711**	**0.907**	**1.647**	**1.698**
N-Succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide	0.013	0.023	0.101	0.081	**0.116**	0.084
N-CBZ-L-Gly-L-Gly-L-Leu-*p*-nitroanilide	0.076	**0.207**	0.055	0.010	**0.178**	**0.224**
N-CBZ-L-Ala-L-Ala-L-Leu-*p*-nitroanilide	0.099	**0.131**	0.073	0.086	**0.128**	**0.152**
N-Succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide	**0.729**	**0.425**	**0.166**	**0.128**	**0.709**	**0.712**

[a] – data presented in the Table are OD_{405} values. Enzyme activity values considered significant are set in bold type.


Trypsin-like protease activities [OD405]
UAMH 7955
UAMH 7956
UAMH 9515
ATCC 201044
ATCC 208859
CBS 446.95
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0.8
0.6
0.4
0.2
0.0
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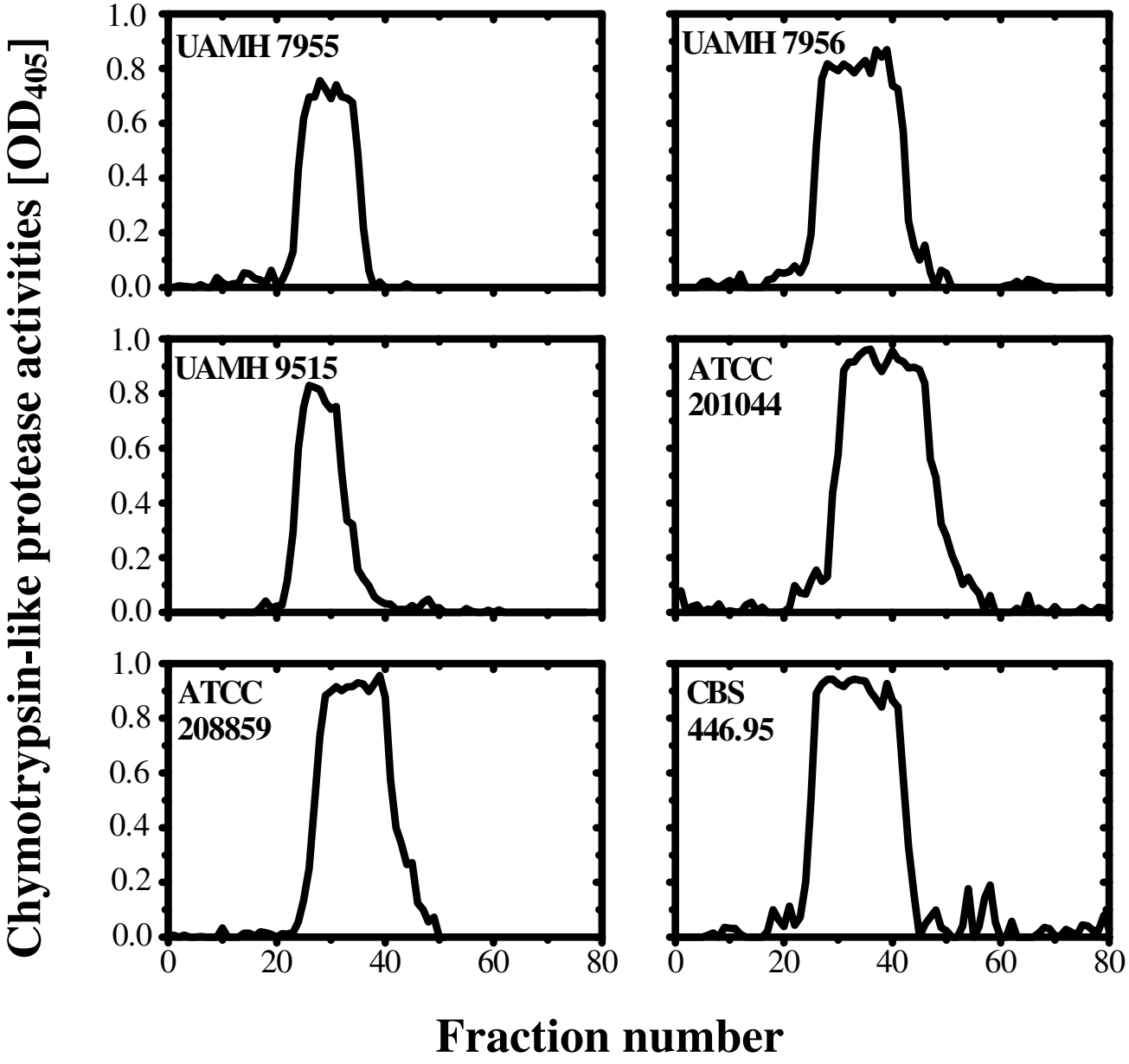
80
Fraction number


Chymotrypsin-like protease activities [OD405]
UAMH 7955
UAMH 7956
UAMH 9515
ATCC 201044
ATCC 208859
CBS 446.95
1.0
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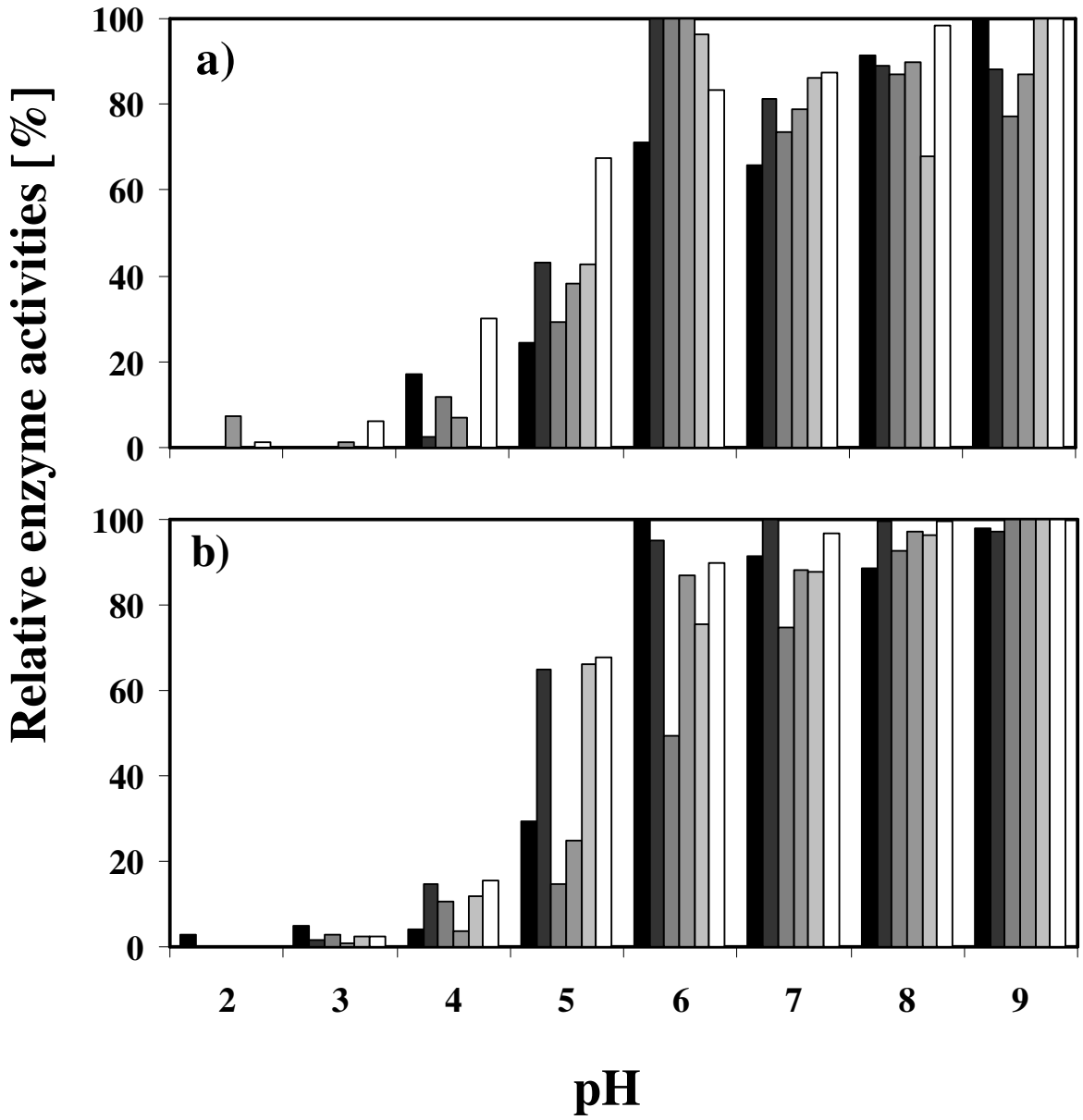
0.4
0.2
0.0
0
20
40
60
80
Fraction number


a)
b)
100
80
60
40
20
0
Relative enzyme activities [%]
2
3
4
5
6
7
8
9
pH