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PROTEOLYTIC SYSTEMS IN THE AQUATIC FUNGUS *ALLOMYCES*

BY

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ABSTRACT

Proteolytic systems in the aquatic fungus *Allomyces*. - In the exponentially growing vegetative cells of *Allomyces arbuscula* we have identified three more enzyme activities using synthetic peptide substrates. These were in addition to two identified previously as Ca^{2+}-dependent proteases (CDP I and CDP II) cleaving peptides at Arg in primary cleavage site (P1 position). One of the three new enzymes specifically cleaved peptides at Arg, another at Ala, Leu or Phe and the third preferred Tyr in P1 position. All these activities were Ca^{2+}-independent. The activity showing preference for Ala in P1 position was different from the similar enzyme from differentiating cells since it was not inhibited by the serine protease inhibitors at concentrations known to inhibit differentiating cell enzyme or trypsin, both serine proteases. These enzymes were not inhibited by classical protease inhibitors.

Key-words: *Allomyces*, Proteolytic enzymes, Substrate specificity.

INTRODUCTION

At the end of the last century, KLEBS (1898-1900), working with aquatic Oomycetes, enunciated the famous principle that the reproductive differentiation in fungi is induced when the balanced growth medium is substituted with dilute salts solution or water. Since then it has generally been accepted that all bacteria and fungi initiate reproductive developmental programme in response to a signal which monitor the threshold of substrate concentration above which vegetative growth is maintained but below which reproductive differentiation is induced. These principles apply to the differentiation in *Allomyces* as well (HATCH, 1935; EMERSON, 1941; TURIAN, 1957). This substrate limitation is mainly restricted to three main elements, carbon, nitrogen and phosphates.

It has also been widely observed that during microbial differentiation many proteins synthesized during growth phase become unstable and are quickly degraded. Against this background we initiated a study on the stability of vegetative proteins during differentiation and observed that they remained stable if the mycelia were transferred to growth medium but became unstable and rapidly degraded when the transfer was made to dilute salts solution, lacking carbon or nitrogen source. Approximately 60% of the proteins synthesized in vegetative phase were degraded during differentiation (OJHA & TURIAN, 1985a). This differential turnover of proteins led us to identify the proteases in the vegetative and differentiating cells.

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Using a nonspecific substrate, azocoll, we could detect only two types of proteolytic activities in *Allomyces*, a Ca^{2+}-dependent in vegetative phase cells and a Ca^{2+}-independent in differentiating cells (OJHA & TURIAN, 1985*b*; OJHA 1996*a*). We could not detect Ca^{2+}-independent activity in the vegetative phase cells. Both Ca^{2+}-dependent and independent enzymes have been purified and characterized (OJHA & WALLACE, 1988; OJHA, 1996*a,b*). On DEAE (DE52)-ion exchange column the Ca^{2+}-dependent enzymes (CDP) were resolved in two peaks of activities, one eluted around 0.04 M (CDP I) and the other between 0.18-0.2 M NaCl (CDP II). These two enzymes have been purified and characterized (OJHA & TURIAN, 1985*b*; OJHA & WALLACE, 1988; OJHA, 1989, 1990, 1996). In SDS-PAGE, CDP I has a relative molecular weight of 39 kDa but CDP II appears as doublet of 43-40 kDa peptide bands. Both enzymes are cysteine proteases, with absolute requirement for Arg in P1 position, the attack site in synthetic peptide substrates (OJHA, 1996*a*; OJHA *et al.*, 1998). All three peptides contain phosphorylable serine residues (OJHA & FAVRE, 1991; FAVRE & OJHA, 1991; OJHA *et al.*, 1994, 1998). The spatial and cellular localization of CDP I is not known but CDP II is cytosolic and localized predominantly in the apical regions of the growing hyphae (HUBER & OJHA, 1994). Two *in vivo* substrates, a protein of 80 kD of unknown function (OJHA, 1990) and β tubuline, seem to be the specific targets (OBREGON *et al.*, unpublished results) of CDP II. CDP I is present in the zoospores and throughout the growth phase. In contrast, CDP II is absent in the zoospores but starts to appear during germination, attains the maximal level during exponential growth and then declines rapidly when the cells are induced to differentiate and finally disappears completely after 1 hour of induction (HUBER & OJHA, 1994; OJHA 1996*b* and unpublished results).

It is inconceivable that *Allomyces* has only two proteases, considering the importance and specificities of these enzymes in various cellular functions. Therefore, we set to identify other proteases using synthetic peptide substrates of defined sequences and in this communication show the presence of additional intracellular enzymes. Some of them are novel considering their resistance to known protease inhibitors.

MATERIAL AND METHODS

Chemicals

Peptide substrates: Bz-Arg-pNA, H-Leu-pNA, Ac-Ala-pNA, Ac-Asp-pNA, Ac-Leu-pNA, Bz-Tyr-pNA, H-Gly-Glu-pNA, H-Gly-Phe-pNA, H-Gly-Arg-pNA, Ac-Pro-Ala-pNA, Bz-Pro-Phe-pNA, Z-Val-Gly-Arg-pNA, P-Glu-Phe-Leu-pNA and Boc-Gly-Gly-Leu-pNA were purchased from Bachem; Z-Arg-Arg-pNA and Boc-Gly-Gly-Leu-pNA from Calbiochem and Tos-Gly-Pro-Arg-pNA and Tos-Gly-Pro-Lys-pNA were from Boehringer Mannheim.

Inhibitors: Leupeptin, antipain, PMSF, 5-DMANSF and 4-APMSF were from Fluka, benzamidin, aprotinin, were from Sigma and E64 was from Boehringer Mannheim. All other chemicals were of analytical grade from Merck.

Experimental strain

The aquatic fungus *Allomyces arbuscula* strain Burma 1D was used as experimental strain; its maintenance and growth conditions have been described earlier (OJHA & TURIAN, 1981, 1985*b*).

Preparation of cellular extract and ion exchange chromatography

Mycelia grown for 18-20 h were harvested, quickly frozen and homogenized to a fine powder in liquid nitrogen. The powder was suspended in the extraction buffer (20 mM MOPS, 2 mM EGTA, 2 mM EDTA, 3 mM $MgCl_2$, 1 mM benzamidin, 0.5 mM PMSF, 215 nM aprotinin, 5 µM leupeptin and 0.25 µg/ml pepstatin), kept in ice for 15 min and then centrifuged at 48 000 x g for 30 min in a Sorvall SS 34 rotor. The supernatant was recovered and a part saved for the determination of the enzyme activities in the cell free extract and the rest chromatographed on DEAE (DE52) ion exchange column pre-equilibrated with the extraction buffer. After thorough washing the adsorbed proteins were eluted in fractions of 2.5 ml against a concentration gradient from 0-0.6 M NaCl, prepared in the extraction buffer. The measurements of proteins and the enzyme activities were done as described earlier (OJHA & WALLACE, 1988; OJHA, 1996*a*,*b*).

RESULTS AND DISCUSSION

Ca^{2+}-dependent and independent enzyme activities in the cell free extract with azocoll and synthetic peptides as substrates

With azocoll as substrate, the major proteolytic activity in the vegetative mycelia was mostly Ca^{2+}-dependent, 4.5 times higher than the basal activity measured in the absence of Ca^{2+} (Tab. I). The specific activity obtained in the absence of Ca^{2+} was only slightly above the background level measured in the controls (reaction mixtures without

TABLE I

Proteolytic activities in the crude extracts of the vegetative and differentiating mycelia

Mycelial type	*Enzyme activity**	
	+ Ca^{2+}	*- Ca^{2+}*
Vegetative	98	22
Differentiaging	--	6595

* µg azocoll degraded/mg protein/min

cell free extracts). In contrast, the proteolytic activity in differentiating cells, 300 times higher than the basal activity in the vegetative cells, was totally Ca^{2+}-independent. Thus, with this non specific substrate the enzyme(s) in differentiating cells were more active than in the vegetative cells.

Using synthetic peptide substrates we could detect 5 major proteolytic activities specific to carboxy terminus of Arg, Ala, Phe, Leu and Tyr in P1 position (SCHECHTER & BERGER, 1965 nomenclature), respectively (Tab. II). The proteolytic activity specific to

TABLE II

Identification of new proteolytic activities with synthetic peptide substrates in the vegetative mycelia of *Allomyces*

Aminoacid at P1 cleavage site	*Specific activity*
Bz-**Arg** pNA	26.8
Z-Arg-**Arg**-pNA	4.2
H-Gly-**Arg**-pNA	15.2
Bz-Phe-Val-**Arg**-pNA	2.2
Z-Val-Gly-**Arg**-pNA	11.3
Tos-Gly-Pro-**Arg**-pNA	40
Tos-Gly-Pro-**Lys**-pNA	24.2
Ac-**Ala**-pNA	11.9
Boc-Ala-**Ala**-pNA	12.6
Ac-Ala-Ala-**Ala**-pNA	11.3
Ac-Pro-**Ala**-pNA	6.4
H-Val-**Ala**-pNA	14.2
Suc-Ala-Ala-Val-**Ala**-pNA	10
H-**Leu**-pNA	2
Ac-**Leu**-pNA	4.5
pGlu-Phe-**Leu**-pNA	2.9
Boc-Gly-Gly-**Leu**-pNA	2.2
Cbz-Gly-Gly-**Leu**-pNA	4.5
Ac-**Phe**-pNA	3.8
Glut-**Phe**-pNA	3.8
H-Gly-**Phe**-pNA	6.8
Bz-**Pro**-pNA	4.8
H-Arg-**Pro**-pNA	8.4
Ac-Pro-**Gly**-pNA	4.2
H-Gly-**Glu**-pNA	3.8
Ac-**Asp**-pNA	3.5
Bz-**Tyr**-pNA	8.4

* µM pNA released/mg protein/min

Arg was Ca^{2+}-depependent as described earlier (OJHA, 1996*a*) and composed of two enzymes, CDP I and CDP II, purified and characterized in detail (OJHA & TURIAN, 1985*b*; OJHA & WALLACE, 1988; OJHA, 1989, 1990; OJHA & FAVRE, 1991; OJHA *et al.*, 1994; HUBER & OJHA, 1994; OJHA, 1996*b*; OJHA *et al.*, 1998). The remaining 4 were new proteolytic activities. These enzyme activities were totally Ca^{2+}-independent (Tab. III). The presence of Ca^{2+} in the reaction mixture appeared to be slightly inhibitory.

TABLE III

Effect of Ca^{2+} on the proteolytic activities in the crude extract of vegetative mycelia of *Allomyces arbuscula* with synthetic peptide substrates

Substrate	*Enzyme activity* *	
	+ Ca^{2+}	– Ca^{2+}
Ac-Ala-pNA	26.6	27.5
H-Val-pNA	4.5	7.7
Succ-Ala-Ala-Val-Ala-pNA	1.3	2.7
Ac-Leu-pNA	8.9	11.8
Ac-Phe-pNA	8.3	12.3
Ac-Asp-pNA	0.8	2.3
BZ-Tyr-pNA	7.7	13.6
Bz-Arg-PNA (CDP I)	22.9	–
BZ-Arg-pNA (CDP II)	22.9	–

* µM pNA released/mg protein/min

Ion exchange chromatography of the crude extract on DEAE (DE52) column and analysis of the fractions for proteolytic activity with azocoll showed characteristic elution CDP I and CDP II (Fig. 1 and OJHA, 1996*b*). There was no measurable basal activity in the absence of Ca^{2+} (Fig. 1). In contrast, when the fractions were assayed with defined peptide substrates containing either Arg, Ala, Leu, Phe or Tyr in P1 position, two peak activities specific to Arg were eluted at around 0.04 and 0.18 M NaCl and required the presence of mM Ca^{2+}, one peak activity specific to Ala, Leu, and Phe eluted at around 0.26 M NaCl; a third peak of enzyme activity, eluted at around 0.28 M NaCl, was specific to peptide containing Tyr in P1 position. The first two activities corresponded to CDP I and CDP II but the later two activities were Ca^{2+}-independent. Since the peak activity eluting around 0.26 M NaCl was active with peptides containing either Ala, Leu, or Phe in P1 position, it can be assumed that the same protein was involved in the hydrolysis of these three peptides. The Tyr specific enzyme activity was probably due to a different enzyme (Fig. 2).

Inhibition of the enzyme with class-specific inhibitors

We have shown that the Ca^{2+}-dependent activities in *Allomyces arbuscula* vegetative cells are due to two enzymes belonging to cysteine protease family (OJHA & WALLACE, 1988; OJHA, 1996*a*). It was of interest to know the affinity of these newly identified enzyme activities to three known classes of proteolytic enzymes, viz. cysteine, serine and aspartate. As shown in Tab. IV, these activities were insensitive to class-specific inhibitors tested. This was not due to the slow reaction of the inhibitors as is known for some enzymes, since prolonged preincubation of the extract did not affect the sensitivity of the enzyme to inhibitors. The substrate specificity of one of the Ca^{2+}-independent proteases from the vegetative cells was similar to the enzyme purified from differentiating cells (OJHA & TURIAN 1985*b*; OJHA, 1996*a*) which was characterized as

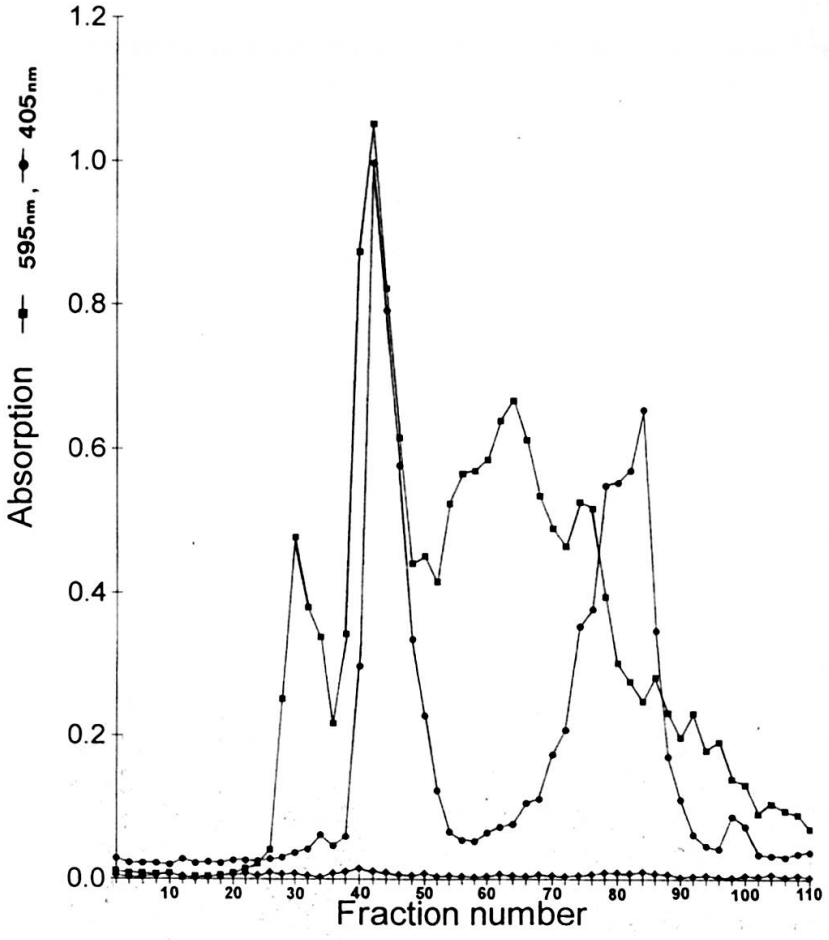

FIG. 1.

Elution profile of the proteolytic activities in the crude extract of *Allomyces arbuscula* from DEAE-cellulose column assayed with azocoll. Protein (■–■), proteolytic activities in the presence of 10 mM Ca^{2+} (●–●) and in the absence of Ca^{2+} (◆–◆).

serine protease specifically cleaving peptides having Ala in P1 position. The sensitivity of the Ala specific enzyme from the vegetative cells was compared to the enzyme from differentiating cells and a classical serine protease, the trypsin, and as shown in Fig. 3, whilst enzymes from differentiating cells and trypsin were highly sensitive to PMSF and the related sulfonyl fluorides, the vegetative enzyme was totally insensitive.

CONCLUSION

We have identified 3 Ca^{2+}-independent intracellular proteolytic enzyme activities from the vegetative mycelia of *Allomyces* in addition to two Ca^{2+}-dependent proteases purified and characterized earlier. These activities can be distinguished on the basis of their ionic charge. One peak activity is specific to peptide substrates containing Arg, another Ala, Leu or Phe in P1 position and the third to Tyr in P1 position. On the basis of its insensitivity to catalytic specific inhibitors, the enzyme showing preference for substrates having Ala in P1 position was considered different from the protease of same

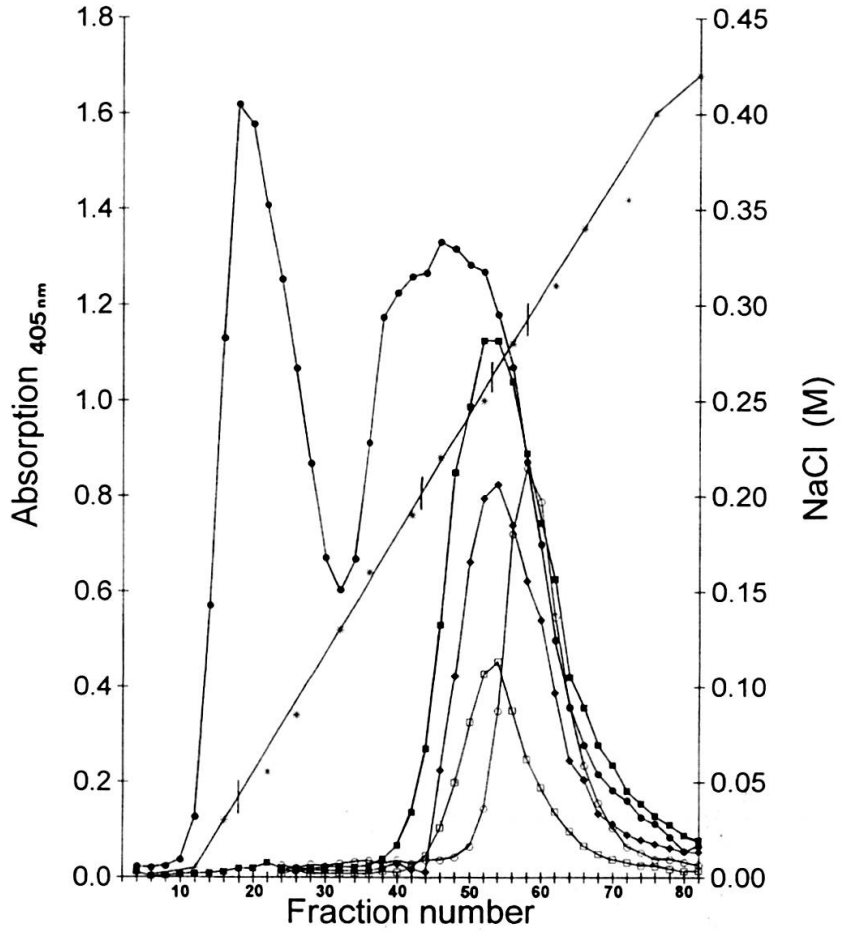

FIG. 2.

Elution profile of the proteolytic activities in the crude extract of *Allomyces arbuscula* from DEAE-cellulose column assayed with synthetic peptide substrates Bz-Arg-pNA (●–●), Ac-Ala-pNA (■–■), Ac-Phe-pNA (◆–◆), Ac-Leu-pNA (□–□), Bz-Tyr-pNA (○–○).

TABLE IV

Effect of class-specific inhibitors on the enzyme activity specific to Ac-Ala-pNA

Class	*Inhibitor*	*Concentration*	*% inhibition of enzyme activity*
Serine	Benzamidine	5 mM	3
	PMSF	1 mM	8
	DCIC	50 μM	2
	STI	50 μM	0
	*Bacitracin	5 mM	7
	*Antipain	50 μM	0
	* Leupeptin	10 μM	4
Cysteine	E64	500 μM	2
	$HgCl_2$	1 mM	1
	Iodoacetate	10 mM	56
	Calpain inhibitor	1 μM	5
Aspartate	Pepstatin	100 μM	0
Metalloprotease	EDTA	4 mM	0
	EGTA	4 mM	0

* inhibits both serine and cysteine proteases

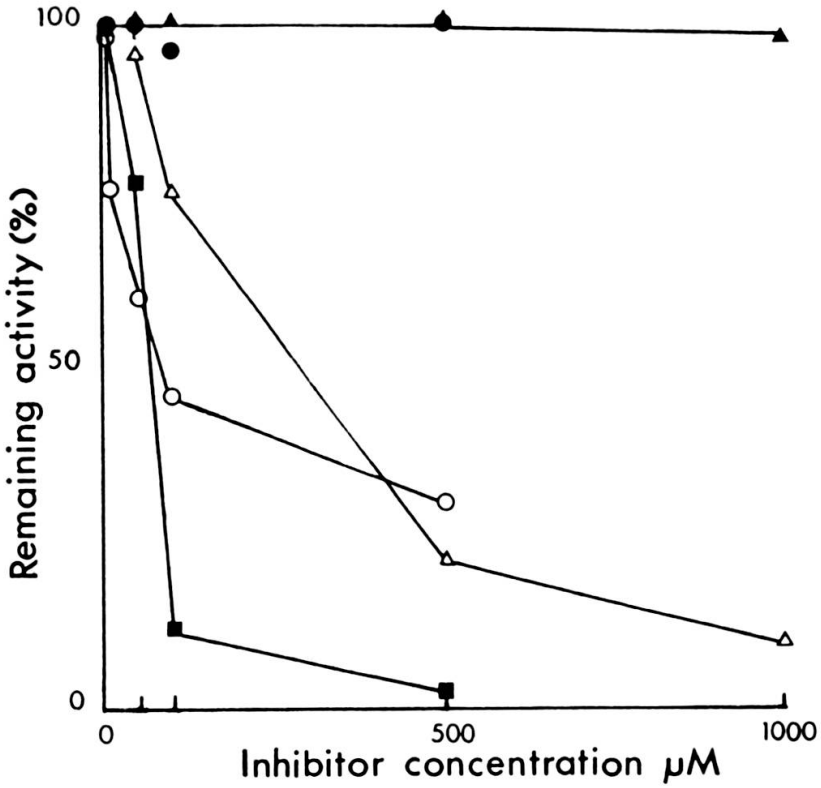

FIG. 3.

Effect of serine protease inhibitors on the catalytic activities of Ac-Ala-pNA-specific enzymes from vegetative (●–● DCIC, ▲–▲ PMSF), differentiating mycelia (■–■ PMSF) and trypsin (○–○ DCIC, Δ–Δ PMSF).

specificity purified from the differentiating cells which has a PMSF sensitive catalytic serine residue. Further information will come when the enzymes will be purified and their properties compared.

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RÉSUMÉ

SYSTÈMES PROTÉOLYTIQUES CHEZ *ALLOMYCES*, UN CHAMPIGNON AQUATIQUE

Nous avons mis en évidence 5 enzymes protéolytiques intracellulaires dans les hyphes végétatifs d'*Allomyces arbuscula*. Deux de ces enzymes, identifiés et décrits auparavant, requièrent du Ca^{2+} en concentration mM pour leur activité catalytique. Ces enzymes reconnaissent l'Arg en position primaire d'hydrolyse du substrat. Trois

nouveaux enzymes avec des spécificités primaires pour: a) Arg, b) Ala, Leu, Phe et c) Tyr ont été identifiés en utilisant des peptides synthétiques. L'activité catalytique de ces enzymes est Ca^{2+} indépendante. Quelques propriétés de ces enzymes sont présentées.

Mots-clés: *Allomyces*, enzymes protéolytiques, spécificité du substrat.

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