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The *Physarum polycephalum php* gene encodes a unique cold-adapted serine-carboxyl peptidase, physarolisin II

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Abstract The *php* gene from a true slime mold, *Physarum polycephalum*, is a late-replicating and transcriptionally active gene. The deduced amino acid sequence of the gene product is homologous to those of the serine-carboxyl peptidase family, including physarolisin I from the same organism, but lacks the propeptide region. In this study, the protein was expressed in *Escherichia coli* and shown to possess endopeptidase activity with unique substrate specificity. Thus, we named it physarolisin II. The enzyme was revealed to be a kind of cold-adapted enzyme since it was maximally active at 16–22°C. The active enzyme was markedly unstable due to rapid autolysis ($t_{1/2}$ = ~5 min, at 18°C). At higher temperature, the enzyme was less active but more stable, despite the fact that no gross conformational change was observed by circular dichroism spectroscopy.

Key words: Autolysis; Cold-adapted enzyme; Physarolisin II; Serine-carboxyl peptidase; True slime mold; *Physarum polycephalum*

1. Introduction

In eukaryotes, transcriptionally active genes are usually replicated early in the S-phase [1]. The *php* gene (GenBank X64708) from a slime mold, *Physarum polycephalum*, is an exception; it is expressed specifically in the plasmodium, a naturally synchronous multinucleic cell differentiating from the conjugated haploid myxamoebae, and replicated late in the S-phase [2]. The molecular mechanism for such regulation and the function of the gene product remain unknown. Recently, a group of enzymes whose amino acid sequences are homologous (identities, 10–20%) to that of the *php* gene product (365 residues), including sedolisin from *Pseudomonas* sp. 101 [3], sedolisin-B from *Xanthomonas* sp. T-22 [4], kumamolisin from *Bacillus* novo sp. MN-32 [5], ScpA from *Alicyclobacillus sendaiensis* [6], aorsin from *Aspergillus oryzae* [7], physarolisin from *P. polycephalum* [8], and CLN2 from human [9], was found and shown to form a new peptidase family called serine-carboxyl peptidases (MEROPS S53) [10,11]. The enzymes in this family contain an essential serine residue and carboxyl groups to form a catalytic triad at the active site, despite the fact that they function optimally under acidic conditions like ordinary aspartic peptidases. Though the amino acid sequence of the *php* gene product is much shorter than those of the homologs apparently due to lack of the propeptide region, the residues constituting a catalytic triad and a Ca^{2+}-binding site appear to be conserved. This suggests that the protein might be an enzyme belonging to the serine-carboxyl peptidase family.

In this study, the *php* gene product was expressed in *Escherichia coli* and shown to act as an endopeptidase under acidic conditions with unique substrate specificity and to have an essential serine residue. Since the enzyme is the second serine-carboxyl peptidase found in *P. polycephalum*, we named it physarolisin II. Interestingly, it was shown to be a kind of cold-adapted enzyme, having its optimal temperature at 16–22°C. This temperature range is similar to that which is optimal for growth of *P. polycephalum* [12]. At the optimal temperature, however, it is markedly unstable due to autolysis, whereas above that temperature, it is much less active but more stable. These changes in activity and stability, however, do not appear to be due to a gross conformational change of the enzyme.

2. Materials and methods

2.1. Materials

The cDNA library of the plasmodia of *P. polycephalum* was a generous gift from Dr. A. Nakamura (Department of Medicine, Gunma University). KPIEFF(NO_2)RL (Lys-Pro-Ile-Glu-Phe-Phe(4-NO_2)-Arg-Leu) was synthesized by a conventional Fmoc solid-phase peptide synthesis method. Synthetic oligonucleotides were obtained from Sawady Technology. Ala-Ala-Phe-CH_2Cl, Ala-Ala-Phe-4-methylcoumaryl-7-amide, DAN (diazoacetyl-D,L-norleucine methyl ester), DFP (diisopropylfluorophosphate), EDTA (ethylenediamine tetraacetic acid) and EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) were from Sigma. All other reagents were of analytical grade.

2.2. Construction of the expression plasmid

The *php* gene lacking the region coding for the putative signal sequence (residues 2–16) was amplified from the cDNA library of the plasmodia of *P. polycephalum* by polymerase chain reaction using the primers GGAATTCCATATGGGTGTTCGCCACCCCCA and ATGCATCTCGAGCTCAGAACCTATCCAAGC, and an EX Taq DNA polymerase (Takara). The amplified fragment was inserted into the *Nde*I–*Xho*I site of the pET21a(+) plasmid (Novagen) so as to produce the protein with a C-terminal Leu-Glu-His-His-His-His-His-His sequence (His-tag). The resulting plasmid was designated pET21/php.

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Abbreviations: DAN, diazoacetyl-D,L-norleucine methyl ester; DFP, diisopropylfluorophosphate; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; KPIEFF(NO_2)RL, Lys-Pro-Ile-Glu-Phe-Phe(4-NO_2)-Arg-Leu

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Table 1
The cleavage rates and specificity of physarolisin II toward peptides

Substrate	Cleavage rate (min^{-1})	Amino acid sequence and cleavage site
Oxidized insulin B chain	17.7	FVNQHC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFFYTPKA
Glucagon	1.8	HSQGTFSDYSKYLDSRRAQDFVQWLMNT
Substance P	1.6	RPKQQFFGLM–NH_2
KPIEFF(NO_2)RL	0.26	KPIEFF(NO_2)RL

Each peptide (5.0 μg) was incubated with the enzyme (1.0 μg) for 10 min at 18°C in 100 μl of 100 mM sodium citrate buffer, pH 4.2. The reaction mixtures were then applied to an HPLC apparatus (1100 series, Agilent Technology) with a TSKgel ODS-120T column (2.2×150 mm, Tosoh) connected to an LCQ™-DUO mass spectrometer (ThermoQuest). Amino acid sequences of the peptides produced were determined from the mass spectra of the original and fragmented ions by using an Xcalibur Bioworks 1.0 software installed in the apparatus, as described in [22]. The cleavage rates were estimated based on the rates of disappearance of the original peptide. Large and small arrowheads indicate the major and minor cleavage sites, respectively. Cleavages at the major sites were at least 10 times faster than those at the minor sites. The amino acid sequences are shown using one-letter code for amino acids.

2.3. Expression and purification of the protein

E. coli BL21(DE3) cells harboring pET21/php were grown in 500 ml of Luria broth containing 50 μg/ml ampicillin at 37°C with gentle shaking to $A_{600}=0.8$ and then the protein expression was induced by adding 1 mM isopropyl-β-thiogalactopyranoside (final concentration). After cultivation for a further 40 h at 15 °C, the cells were harvested by centrifugation, suspended in 20 ml of buffer A (20 mM Tris–Cl and 500 mM NaCl, pH 7.9) containing 5 mM imidazole and frozen in liquid nitrogen. The frozen cell suspension was then thawed, sonicated and centrifuged (10 000×*g* for 30 min). The supernatant was applied to a His-Bind® column (15×25 mm, Novagen) equilibrated with buffer A containing 5 mM imidazole. After washing the column by six column volumes of buffer A containing 60 mM imidazole, the recombinant protein was eluted with six volumes of buffer A containing 240 mM imidazole. The protein fraction was desalted by a PD10 column (Amersham Bioscience) equilibrated with buffer A and used for further experiments. Protein concentration was determined by using a BCA™ protein assay reagent kit (Pierce).

2.4. Enzyme assay

The enzyme activities toward peptides were assayed as described in each table and figure legend. Those toward hemoglobin, casein, azocoll and Ala-Ala-Phe-4-methylcoumaryl-7-amide were assayed as described in [6,8,13].

2.5. Other methods

N-terminal amino acid sequencing was performed in a Perkin Elmer model 477 amino acid sequencer. Circular dichroism (CD) spectra were measured in a Jasco J-720 spectropolarimeter as described in the legend to Fig. 3.

3. Results

3.1. Overexpression and purification of the php gene product

The recombinant *php* gene product, of which the N-terminal putative signal sequence (residues 2–16), predicted according to Heijne et al. [14], was removed and a C-terminal His-tag was added, was overexpressed as a soluble 38 kDa protein. N-terminal amino acid sequencing gave the results: Gly-Val-Arg-His-Pro-, which confirmed that it corresponded to the *php* gene product (Δ1–16). The N-terminal methionine was missing, which is thought to have been removed by an intracellular methionyl aminopeptidase [15]. The protein was then purified by His-Bind® chromatography, desalted and used for the further experiments. On the other hand, when the recombinant protein with the N-terminal putative signal sequence was expressed in the same manner, it formed an inclusion body (data not shown), indicating that the signal peptide was not removed by *E. coli* signal peptidase [16] and interfered with the correct folding of the protein in *E. coli* cells.

3.2. Endopeptidase activity and substrate specificity of physarolisin II

The protein was found to hydrolyze oxidized insulin B chain, glucagon, substance P and KPIEFF(NO_2)RL at pH 4.2 and 18°C (Table 1). The cleavage rate toward oxidized insulin B chain was about 10 times higher than those toward glucagon and substance P, and about 70 times higher than that toward KPIEFF(NO_2)RL. Since the protein was shown to possess endopeptidase activity and since it is the second serine-carboxyl peptidase from *P. polycephalum*, we named it 'physarolisin II'. In this context, we also propose to rename the first enzyme 'physarolisin' [8] as 'physarolisin I' to distinguish between the two enzymes. Physarolisin II, however, did not hydrolyze hemoglobin, casein, azocoll and Ala-Ala-Phe-4-methylcoumaryl-7-amide, a good substrate for tripeptidylpeptidase including CLN2 [17], at pH 3.0–7.5 and 18–37°C. As for the cleavage specificity of physarolisin II, bulky hydrophobic or aromatic residues, such as Leu, Phe and Tyr residues, were notably located at either or both of the P1 and P′1 positions of the major cleavage sites.

3.3. Effects of inhibitors on physarolisin II

The enzyme activity was inhibited by DFP, but not by pepstatin (Table 2), indicating that the enzyme is a member of the serine-carboxyl peptidases which contain an essential serine residue in the catalytic site [8,17]. The insensitivity to Ala-Ala-Phe-CH_2Cl, a potent inhibitor of tripeptidylpeptidase including CLN2 [17], is consistent with the fact that the enzyme did not hydrolyze the tripeptidylpeptidase substrate as described above. In these respects, the enzyme resembles phys-

Table 2
Effects of inhibitors on the activity of physarolisin II

Inhibitor	Concentration (mM)	Inhibition (%)
Ala-Ala-Phe-CH_2Cl	10	0
DAN[a]	0.2	0
DFP[b]	10	76
	5	50
	0.5	23
EDTA	10	0
EGTA	10	0
Pepstatin	0.01	0

The enzyme (7.3 μg) was incubated with oxidized insulin B chain (5.0 μg) for 10 min at 18°C in 100 μl of 100 mM sodium citrate buffer, pH 4.2, in the presence of each inhibitor. The reaction mixtures were then analyzed as described in Table 1.
[a]The enzyme was pre-incubated with DAN for 10 min at pH 4.2 and 37°C in the presence of 0.13 mM $CuSO_4$.
[b]The enzyme was pre-incubated with DFP for 10 min at pH 4.2 and 37°C.

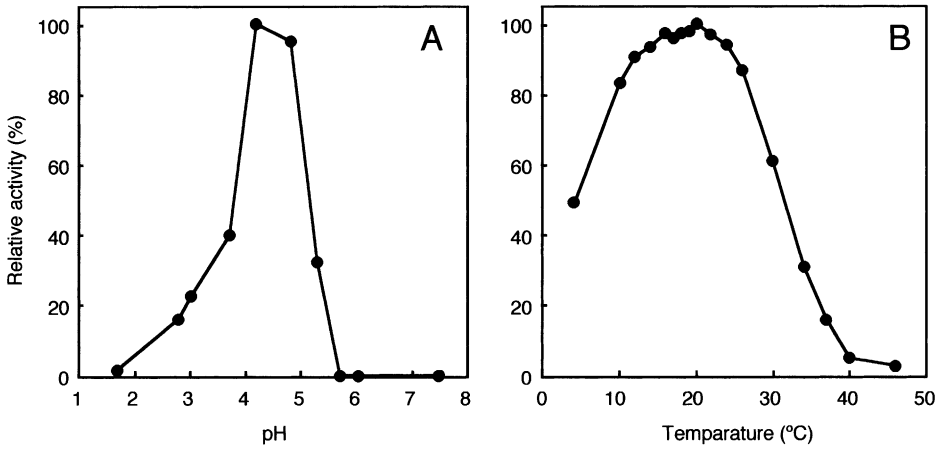

Fig. 1. pH and temperature dependences of the activity of physarolisin II. The enzyme (7.3 μg) was incubated with oxidized insulin B chain (5.0 μg) for 10 min in 100 μl of 100 mM sodium citrate buffer except for the following variations. A: The pH was varied using the following buffers: HCl–KCl buffer for pH 1.7, sodium citrate buffers for pH 3.0–5.7, and sodium phosphate buffers for pH 6–7.5. B: The temperature was varied. The reaction mixtures were then analyzed as described in Table 1.

arolisin I. However, it was not inhibited by DAN, unlike physarolisin I.

3.4. Optimal pH and temperature of physarolisin II

The optimal pH of the enzyme activity toward oxidized insulin B chain was shown to be 4.2 (Fig. 1A). This is comparable with those of the homologs [3–8,17], except for physarolisin I, which is optimally active at pH 1.7 toward hemoglobin [13]. Interestingly, the enzyme activity was maximum at 16–22°C (relative activity, 95–100%) and decreased above this temperature (Fig. 1B).

3.5. Autolysis of physarolisin II

Upon incubation at pH 4.2 and 18°C, the band of the enzyme (Δ1–16) disappeared immediately ($t_{1/2}$ = ~5 min) as examined by SDS–PAGE (Fig. 2). During the incubation, a protein band with a slightly lower molecular weight was observed after 5 min and then disappeared at a rate similar to that of the original enzyme. The protein in the lower band was shown by amino acid sequencing to have the N-terminal sequence Phe-Lys-Ile-Ala-Gly-, indicating that it is the enzyme (Δ1–22) lacking an additional six residues from the N-terminus. The disappearance of the enzyme was partially blocked by pre-incubating the enzyme with 10 mM DFP at pH 4.2 and 37°C for 10 min. On the other hand, the protein was much more stable at 37°C at pH 4.2 ($t_{1/2}$ = ~6 h) and very stable at pH 7.5.

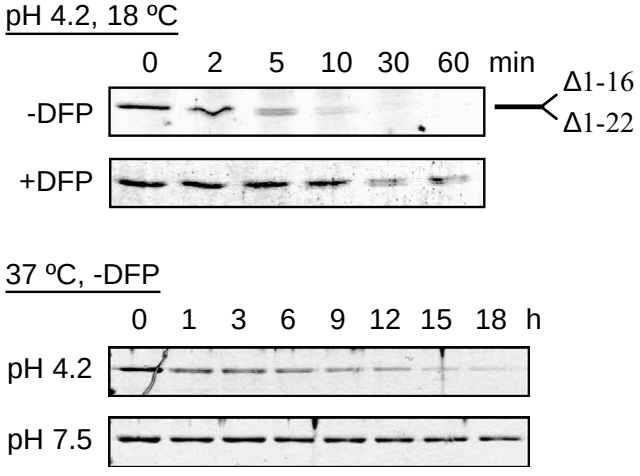

Fig. 2. Time course of autolysis of physarolisin II. The enzyme (12 μg) was incubated in 300 μl of 100 mM sodium citrate buffer, pH 4.2, or sodium phosphate buffer, pH 7.5, at the indicated temperature and pH. The DFP-treated enzyme was prepared by pre-incubating the enzyme with 10 mM DFP for 10 min at 37°C. At appropriate intervals, a 20 μl aliquot was withdrawn and mixed with 4 μl of 6× SDS sample buffer. These samples were subjected to SDS–PAGE and proteins were detected by Coomassie brilliant blue R250 staining.

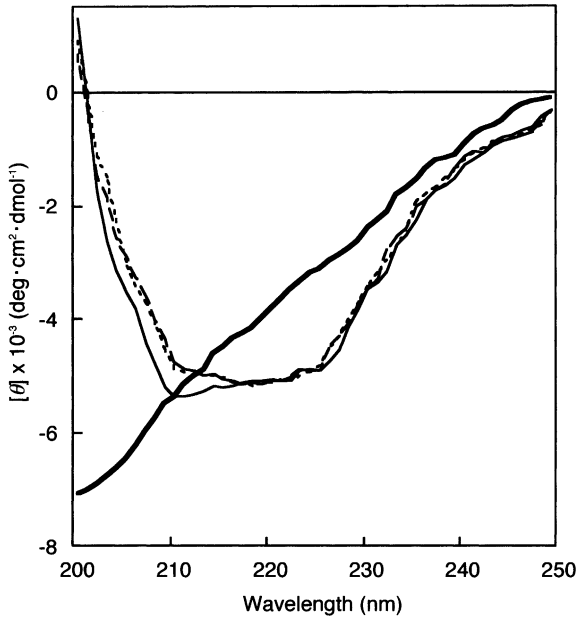

Fig. 3. CD spectra of physarolisin II. Each spectrum was the average from eight measurements using a 0.1 cm cuvette at a protein concentration of 2.8 μM in 20 mM Tris–Cl buffer, pH 7.9, or 100 mM sodium citrate buffer, pH 4.2, at 37°C or 20°C. The DFP-treated enzyme was prepared by pre-incubating the enzyme with 10 mM DFP for 10 min at 37°C and pH 4.2. The spectra of the enzyme at 37°C at pH 7.9 and pH 4.2 are shown with a bold and a thin solid line, respectively. Those of the DFP-treated enzyme at pH 4.2 at 37 and 20 °C are shown with a broken and a dotted line, respectively.

3.6. CD spectra of physarolisin II

To investigate the change of the secondary structures of the enzyme under different pH and temperature conditions, far-UV CD spectra of the intact and DFP-treated enzymes were measured (Fig. 3). The spectrum of the enzyme at pH 7.9 and 37°C represented the denatured structure. This spectrum was rapidly changed to that apparently representing the folded structure by decreasing pH to 4.2. Treatment of the enzyme with 10 mM DFP for 10 min at pH 4.2 and 37°C resulted in little change in the spectrum. Furthermore, the spectra of the DFP-treated enzyme at pH 4.2 at 37°C and 20°C were almost identical.

4. Discussion

In this study, the recombinant *php* gene product was expressed in *E. coli* and was shown to possess endopeptidase activity toward various peptide substrates. Moreover, the enzyme was found to be a serine-carboxyl peptidase as judged from the inhibition by DFP and the pH–activity profile with an acidic pH optimum. This is consistent with the fact that the primary structure of the enzyme contains the residues apparently corresponding to those commonly present in the catalytic site and Ca^{2+}-binding site of other serine-carboxyl peptidases. Since the enzyme is the second serine-carboxyl peptidase identified in *P. polycephalum*, it should be reasonable to rename the first enzyme 'physarolisin' as 'physarolisin I' and to call the present enzyme 'physarolisin II'. [2,8]. These enzymes, however, are quite different from each other in the overall amino acid sequences (identity, 16%). It is the first time that two distinct serine-carboxyl peptidases have been shown to be present in one organism.

Physarolisin II is distinct in several properties from the hitherto-known serine-carboxyl peptidases, including physarolisin I. First, the enzyme is most active at 16–22°C, indicating that it is a kind of cold-adapted enzyme [18–20]. It is the first cold-adapted enzyme found among the serine-carboxyl peptidases. *P. polycephalum* grows optimally in a similar temperature range [12], and therefore the enzyme appears to be well adapted to this growing temperature. Interestingly, the stability of the enzyme is also highly dependent on temperature; the enzyme is unstable at 18°C due to autolysis, but not at 37°C. These results suggest that the activity and amount of the enzyme in the mold is strictly regulated by environmental temperature. The rapid autolysis at 18°C seems to indicate that the enzyme possesses a fairly flexible conformation at the cold temperature, which should also be favorable for expression of the enzyme activity under such conditions. This assumption is consistent with the fact that the cold-adaptation of an enzyme is generally achieved through such a flexible structure of the enzyme molecule [18–20]. On the other hand, it is notable that the enzyme is less active but more stable at higher temperatures, such as 37°C, despite the fact that no gross conformational change was observed between the structures at 37 and 20°C in the CD spectroscopy. This is in sharp contrast with the fact that other cold-adapted enzymes are usually heat-labile at a moderate temperature, where they tend to lose their activity due to denaturation. The molecular mechanisms of these features of the present enzyme are not clear at present, and further structural studies, such as X-ray, nuclear magnetic resonance and calorimetric analyses, will be necessary to clarify them.

Secondly, the enzyme is unique in that it has no propeptide corresponding to those in the other known serine-carboxyl peptidases. The latter possess a long N-terminal propeptide of approximately 170–240 residues. During autolysis, the N-terminal six-residue segment appeared to be removed. At present, it is not clear whether this segment acts as the propeptide. The expressed and purified enzyme preparation at pH 7.9 had a denatured form as examined by CD spectroscopy. However, the enzyme was found to be folded very quickly when exposed to pH 4.2 to generate the enzymatic activity. It will be interesting to elucidate how the enzyme can fold into the proper tertiary structure without a long propeptide like those of its homologs; sedolisin was reported to need the full-length propeptide of 215 residues for folding to the active enzyme [3].

Thirdly, the substrate specificity of the enzyme is unique in that it does not hydrolyze hemoglobin and casein; other serine-carboxyl peptidases, such as sedolisin [3], sedolisin-B [4], kumamolisin [5], and physarolisin [8], are known to hydrolyze either of the proteins. Furthermore, since the enzyme lacks the activity toward Ala-Ala-Phe-4-methoxycoumaryl-7-amide and azocoll, it is neither a tripeptidylpeptidase like CLN2 [21] nor a collagenolytic enzyme like ScpA and kumamolisin [6]. It is worth noting that the specificity of the enzyme toward oxidized insulin B chain was also quite different from that of physarolisin I, whose major cleavage sites are Gly^{8}–Ser^{9}, Leu^{11}–Val^{12} and $Cys(SO_3H)^{19}$–Gly^{20} [10]. Therefore, the substrate specificity of physarolisin II seems to be markedly restricted.

The cold-adaptation, as well as the rapid autolysis, restricted substrate specificity and unique timing of the gene replication, together suggest that the enzyme might play a very unique role in vivo, possibly regulated by the change of environmental temperature, through processing or hydrolyzing a specific or a limited kind of substrate(s). Thus, the enzyme stands out in both functional and structural features among the serine-carboxyl peptidase family.

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