Comparative Study on the Specificities of Several Fungal Aspartic and Acidic Proteinases towards the Tetradecapeptide of a Renin Substrate

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The tetradecapeptide of a renin substrate, DRVYIHPFHLVYS, was used as a substrate for assaying several fungal aspartic and acidic proteinases in the acidic pH range. Aspartic and acidic proteinases from Phycomycetes, *Mucor* and *Rhizopus*, and Deuteromycotina, *Aspergillus* and *Penicillium*, cleaved the tetradecapeptide at its tyrosyl⁴-isoleucyl⁵ (Y⁴-I⁵), histidyl⁶-prolyl⁷ (H⁶-P⁷) and leucyl¹¹-valyl¹² (L¹¹-V¹²) bonds in the acidic pH range, while acidic proteinases type B and type A-I from *Scytalidium lignicolum*, and those from *Cladosporium* and Basidiomycetes, *Pycnoporus sanguineus*, and the yeast, *Rhodotorula glutinis*, showed slightly different specificities towards the tetradecapeptide. Pepsin primarily cleaved the valyl³-tyrosyl⁴ (V³-Y⁴) and leucyl¹⁰-leucyl¹¹ (L¹⁰-L¹¹) bonds. All of the aspartic and acidic proteinases of fungal origin tested in the present study have different specificities from that of pepsin.

Of the aspartic proteinases, pepsin (EC 3.4.23.1) acts on hemoglobin and the synthetic substrate, acetyl-L-phenylalanyl-diiodotyrosine (Ac-FY-I₂).¹⁾ Pepsin exhibits a milk-clotting action at pH 5.3. While the aspartic proteinase from *Aspergillus saitoi* (EC 3.4.23.6) does not hydrolyze Ac-FY-I₂, it is capable of activating trypsinogen and chymotrypsinogen in the acidic pH range.²⁾ The proteinase was inactive as to the milk clotting. The digestion of oxidized insulin B-chain by the *Aspergillus* aspartic proteinase is not similar to that in the case of human pepsin 5, human pepsin 3 and porcine pepsin C, but is similar to that in the case of cathepsin D (EC

3.4.23.5) from human erythrocytes.^{3,4)} In the previous study,⁵⁾ preparations of *Aspergillus* aspartic proteinase and *Scytalidium lignicolum* acidic proteinase B were shown to cleave the H⁶-P⁷ bond of proangiotensin (formerly designated as angiotensin I) at pH 3.0 and 2.2, respectively. An additional cleavage site of proangiotensin for the two aspartic and acidic proteinases was the Y⁴-I⁵ bond. On the other hand, pepsin slightly hydrolyzed the valyl³-tyrosyl⁴ (V³-Y⁴) bond.

The tetradecapeptide, DRVYIHPFHLVYS, is a specific substrate used for the determination of renin (EC 3.4.23.15) activity.⁵⁾ The cleavage site of renin is the leucyl¹⁰-

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leucyl¹¹(L¹⁰-L¹¹) bond. In the present study, we expected that the tetradecapeptide would be a useful substrate for proteinases instead of the oxidized insulin B-chain.

MATERIALS AND METHODS

Materials. The tetradecapeptide (Lot 260116), pro-angiotensin (Lot 310209) and angiotensin (Lot 260116) were purchased from Peptide Institute Inc., Ina, Mino-shi, Osaka. Dansyl (DMS) chloride was obtained from Tokyo Kasei Kogyo Co., Ltd. The polyamide sheets were from Seikagaku Biochemicals, Tokyo.

The crude enzyme preparations of *Aspergillus oryzae* No. 406, *Aspergillus sojae* KS and *Penicillium duponti* K-1014 were kind gifts from Dr. H. Motai of Kikkoman Co., Ltd., Noda. *Mucor racemosus* No. 50 was a gift from Dr. K. Ahiko of Snow Brand Co., Ltd., Kawagoe. The newlase preparation of *Rhizopus* sp. was a gift from Dr. H. Tanaka of Amano Pharmaceutical Co., Nagoya.

Crystalline porcine pepsin was purchased from General Biochemical Inc. (Lot 3036). Bovine spleen cathepsin D was obtained from Sigma (Lot 26C-8100).

Aspartic and acidic proteinases. Purification of the *Aspergillus oryzae* aspartic proteinase was performed by the method of Davidson *et al.*⁷⁾ Further purification of the proteinase was performed by the isoelectric focusing method described. Protein was determined from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 16.0$).⁷⁾ Purification of the *Aspergillus sojae* aspartic proteinase I was performed by the method described previously.⁴⁾ Purification of the *Aspergillus saitoi* aspartic proteinase was performed by a previously described method.^{3,8)} Protein was determined from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 13.15$).²⁾

Purification of the *Penicillium duponti* acidic proteinase was performed by the method of Hashimoto *et al.*⁹⁾ Protein was determined from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 11.45$).¹⁰⁾

Purification of the acidic proteinase from *Mucor racemosus* No. 50 was performed according to a previous paper.¹¹⁾ Purification of the aspartic proteinase from *Rhizopus* sp. was performed according to a previous paper.¹²⁾ Protein was determined from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 12.9$).¹²⁾

Purification of acidic proteinases type B and type A-1 from *Scytalidium lignicolum* was performed as described in a previous paper.¹³⁾ Protein was estimated from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 11.0$).¹⁴⁾

Purification of the acidic proteinase from *Pycnoporus sanguineus* (formerly designated as *Trametes sanguinea* or *Pycnoporus coccineus*) was performed according to a previous paper.¹⁵⁾ Protein was determined from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 15.0$). Purification of the *Cladosporium* sp. acidic proteinase was performed accord-

ing to a previous paper.¹⁶⁾ Protein was determined from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 11.7$).¹⁷⁾ Purification of the acidic proteinase from the yeast, *Rhodotorula glutinis* K-24 was performed as previously described.¹⁸⁾ Protein was estimated from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 14.0$).¹⁹⁾

Purification of pepsin was performed according to a previous paper by Kaiser and Nakagawa.²⁰⁾ The protein concentration in the case of porcine pepsin was determined from the absorbance at 280 nm ($E_{1\text{cm}}^{1\%} = 14.1$).²¹⁾ Bovine spleen cathepsin D was used without further purification.

Separation and identification of peptides from the digests of the tetradecapeptide, proangiotensin and angiotensin. The tetradecapeptide (1.14 μmol , 2 mg), proangiotensin (1.54 μmol , 2 mg) or angiotensin (1.91 μmol , 2 mg) was dissolved in 10 ml of dilute HCl, 8 nkat of an aspartic or acidic proteinase was added to the solution, and then the pH was adjusted to pH 2.2~3.2 with dilute HCl. Each mixture was incubated at 30°C for 3 hr. A few drops of concentrated NH₄OH were added to inactivate the enzyme. Samples of the hydrolysate were cooled to -20°C, and then the frozen digest was lyophilized.

The freeze-dried digest was then dissolved in 100 μl of 1 M NH₄OH and then separated on Toyo filter paper, in the first dimension, by high voltage paper electrophoresis in pyridine-acetic acid-water, 10:0.4:90 (v/v), at pH 6.5 for 150 min at 45 V/cm. After drying at room temperature, ascending chromatography was carried out with a solvent system of *n*-butanol-acetic acid-water, 4:1:2 (v/v), for 60 hr. The peptide maps were stained with 0.02 or 0.2% ninhydrinacetone reagent and 0.1% *p*-nitrobenzene sulfonic acid solution (Paury's reagent). Ninhydrin positive zones and Paury's reagent positive zones were cut out from the sheet and then the peptides were washed with acetone and then eluted with water. The eluates were freeze-dried *in vacuo*.

Amino acid analysis. Freeze-dried peptides were dissolved in 2 ml of 6 M HCl and then hydrolyzed at 110°C for 24 hr. Hydrolysates of peptides were examined with a Hitachi amino acid analyzer, model KLA-3A or 835-30.

Determination of N-terminal amino acids of the peptides. The N-terminal amino acids of the purified peptides were determined by the DNS-method.²²⁾

Determination of C-terminal amino acids of the peptides. The release of the C-terminal amino acids from the peptides was performed using *Penicillium janthinellum* acid carboxypeptidase^{23,24)} at pH 3.7 and 30°C. The enzyme is now commercially available from Peptide Institute, Inc., Ina, Mino-shi, Osaka (Code 3502).

Estimation of the extents of hydrolysis of the various peptide bonds. The extents of hydrolysis of the individual

peptide bonds which were split were then calculated by summation of the yields of the peptides located on either side of each opened bond.

RESULTS

Figure 1 is a summary of the specificities of the aspartic proteinase from *Aspergillus oryzae* towards angiotensin, proangiotensin and the tetradecapeptide at pH 3.0 and 30°C on 3 hr-hydrolysis. The results show that the Y⁴-I⁵ bonds in angiotensin, proangiotensin and the tetradecapeptide were easily hydrolyzed. Although the H⁶-P⁷ bond is not a usual peptide bond, enzymatic hydrolysates of the H⁶-P⁷ bonds of proangiotensin and the tetradecapeptide was observed, and two peptides from each (I⁵H⁶ and P⁷FHL¹⁰, and I⁵H⁶ and P⁷FHLL¹¹) were obtained with the aspartic proteinase from *Aspergillus oryzae*. The extent of hydrolysis of the H⁶-P⁷ bond was about 50~60% of that of the Y⁴-I⁵ bond. The aspartic proteinase from *Aspergillus oryzae* could not split the H⁶-P⁷ bond in angiotensin. This aspartic proteinase also cleaved the L¹¹-V¹² bond in the tetradecapeptide. Thus the aspartic proteinase from *Aspergillus oryzae* exhibited a lower preference for the H⁶-P⁷ bond than for the Y⁴-I⁵ and L¹¹-V¹² bonds.

Figure 2 shows the specificities of aspartic or acidic proteinases from two species of Pycomycetes, *Mucor racemosus* and *Rhizopus* sp., and three species of Deuteromycotina, *Aspergillus sojae*, *Aspergillus saitoi* and *Penicillium duponti*. These aspartic or acidic proteinases hydrolyzed three bonds, Y⁴-I⁵, H⁶-P⁷ and L¹¹-V¹². On hydrolysis by the proteinases from *Rhizopus* sp. and *Penicillium duponti*, the relative extents of hydrolysis at L¹¹-V¹² were, respectively, 2- and 3-fold greater than that at Y⁴-I⁵. The relative extent of H⁶-P⁷ hydrolysis by the proteinase from *Penicillium duponti* was great, being about 3-fold greater than that of Y⁴-I⁵ hydrolysis.

Figure 3 shows the specificities of acidic proteinases type B and type A-1 from the Deuteromycotina, *Scytalidium lignicolum*,

FIG. 1. Summary of the Specificities of the Aspartic Proteinase from *Aspergillus oryzae* towards Angiotensin, Proangiotensin and the Tetradecapeptide at pH 3.0 and 30° for 3 hr.

Values in parentheses denote the uncorrected recoveries (μ mol) of peptides. Perpendicular arrows ($\downarrow$) indicate the bonds split, the degree of hydrolysis being as follows: $\downarrow > \downarrow > \downarrow$. The extent of hydrolysis of the Y⁴-I⁵ bond is arbitrarily taken to be 100. Horizontal arrows indicate residues recovered with the following procedures: $\rightarrow$ indicated by the DNS method, $\leftarrow$ amino acid with *Penicillium janthinellum* acid carboxypeptidase; the degree of hydrolysis being as follows; $\leftarrow > \leftarrow > \leftarrow$. 1, angiotensin e/s = 1:13913 (mol/mol); 2, proangiotensin e/s = 1:11224 (mol/mol); 3, the tetradecapeptide e/s = 1:8272 (mol/mol).

towards the tetradecapeptide at pH 2.2 and 3.2, respectively. The type B proteinase clearly hydrolyzed the H⁶-P⁷ and H⁹-L¹⁰ bonds at similar relative rates. The type B proteinase showed lower preference for the Y⁴-I⁵ bond. The type A-1 proteinase clearly hydrolyzed the Y⁴-I⁵, F⁸-H⁹ and L¹⁰-L¹¹ bonds in the tetradecapeptide at pH 3.2 and 30°C for 3 hr.

Figure 4 shows the specificities of acidic proteinases from two species of Basidiomycetes, *Pycnoporus sanguineus* and the yeast, *Rhodotorula glutinis*, and the yeast-like Deuteromycotina, *Cladosporium* sp. The acidic proteinase from *Cladosporium* sp. cleaved Y⁴-I⁵ and L¹¹-V¹² at pH 2.5 and 30°C for 3 hr. Additional cleavage of the bond R²-V³ was noted. The acidic proteinase from *Pycnopo-*

FIG. 2. Summary of the Specificities of the Aspartic or Acidic Proteinases from Two Species of Phycomycetes, *Mucor racemosus* and *Rhizopus* sp. and Three Species of Deuteromycotina, *Aspergillus sojae*, *Aspergillus saitoi* and *Penicillium duponti*.

1, *Mucor racemosus* acidic proteinase, 3-hr incubation at pH 3.0, e/s 1:11 (w/w); 2, *Rhizopus* sp. aspartic proteinase, 3-hr at pH 3.1, e/s 1:172 (mol/mol); 3, *Aspergillus sojae* aspartic proteinase, 3-hr incubation at pH 3.1, e/s 1:172 (mol/mol); 4, *Aspergillus saitoi* aspartic proteinase, 3-hr incubation at pH 2.7, e/s 1:272 (mol/mol); 5, *Penicillium duponti* acidic proteinase, 3-hr incubation at pH 2.5, e/s 1:16 (mol/mol).

rus sanguineus clearly hydrolyzed the Y⁴-I⁵ and L¹¹-V¹² bonds at pH 2.7 and 30°C for 3 hr. That from *Rhotorula glutinis* hydrolyzed the Y⁴-I⁵ and L¹¹-V¹² bonds at pH 2.5 and 30°C for 3 hr. Additional cleavage of the V³-Y⁴ and L¹⁰-L¹¹ bonds was also noted.

Figure 5 shows the specificities of porcine pepsin towards the tetradecapeptide at pH 2.0 and 30°C for 72 hr. Pepsin cleaved the V³-Y⁴ and L¹⁰-L¹¹ bonds. Additional cleavage of the Y⁴-I⁵ and V¹²-Y¹³ bonds was noted. Pepsin

FIG. 3. Specificities of Acidic Proteinases Type B and Type A-1 from *Scytalidium lignicolum*.

1, type B proteinase, 3-hr incubation at pH 2.3 and 30°C, e/s 1:72 (mol/mol); 2, type A-1 proteinase, 3-hr incubation at pH 3.2 and 30°C, e/s 1:251 (mol/mol).

FIG. 4. Summary of the Specificities of the Acidic Proteinases from Two Species of Basidiomycetes, *Pycnoporus sanguineus* and the Yeast, *Rhodotolura glutinis*, and the Yeast-like Deuteromycotina, *Cladosporium* sp.

1, *Cladosporium* sp. acidic proteinase, 3-hr incubation at pH 2.5, e/s 1:165 (mol/mol); 2, *Pycnoporus sanguineus* acidic proteinase, 3-hr incubation at pH 2.7, e/s 1:54 (mol/mol); 3, *Rhodotolura glutinis* acidic proteinase, 3-hr incubation at pH 2.5, e/s 1:104 (mol/mol).

has a different and characteristic specificity compared with fungal aspartic proteinases, but has no ability to hydrolyze the H⁶-P⁷ bond in the tetradecapeptide. Bovine spleen cathepsin D (EC 3.4.23.5) slightly hydrolyzed the Y⁴-I⁵ and F⁸-H⁹ bonds in proangiotensin at pH 3.0

FIG. 5. Summaries of the Specificities of Porcine Pepsin and Spleen Cathepsin D.

1, pepsin, 72-hr incubation at pH 2.0 and 30°C, e/s 1:96 (mol/mol); 2, cathepsin D, 72-hr incubation at pH 3.0 and 30°C, e/s 1:161 (mol/mol).

and 30°C for 72 hr with an enzyme substrate ratio of 1:200 mol/mol (Fig. 5). Although four peptides (D^1RVY^4 , I^5HPFHL^{10} , $D^1RVYIHPF^8$ and H^9L^{10}) were obtained through the action of cathepsin D, the yields were low.

DISCUSSION

The aspartic proteinases from *Aspergillus*^{25,26)} and *Penicillium*¹²⁾ have many properties in common with porcine pepsin and, like pepsin, have aspartic acid residues at their active sites. In previous studies, we found that aspartic proteinases from *Aspergillus* have different specificities towards oxidized insulin B-chain^{3,4)} and proangiotensin,⁵⁾ as compared with pepsin. In the present study we confirmed the specificities of the aspartic and acidic proteinases from *Aspergillus* and of the other fungal origin towards the tetradecapeptide of a renin substrate, which contains 14 amino acid residues with 10 different species.

The aspartic proteinase from *Aspergillus oryzae* could not split the H^6-P^7 bond in angiotensin. This suggests that the P_3' position may be essential for the hydrolysis by an aspartic proteinase. Furthermore, the aspartic proteinase from *Aspergillus* could not hydrolyze the V^3-Y^4 bonds (Figs. 1 and 2). These

results suggest that the P_4 position may be essential for the hydrolysis by the aspartic proteinases from *Aspergillus*. Thus the aspartic proteinases from *Aspergillus* exhibited a lower preference for small peptides and could not hydrolyze any commercial synthetic peptides.²⁾ All of the aspartic or acidic proteinases from Phycomycetes, *Mucor* and *Rhizopus*, and two genera of Deuteromycotina, *Aspergillus* and *Penicillium*, clearly hydrolyzed the H^6-P^7 bond in the tetradecapeptide. In the enzymatic hydrolysates, the dipeptide, I^5H^6 , was recovered with aspartic and acidic proteinases from *Aspergillus oryzae*, *Aspergillus sojae*, *Aspergillus saitoi*, *Penicillium duponti* and *Mucor racemosus*, as shown in Figs. 1 and 2. The acidic proteinase from *Penicillium duponti* showed the highest extent of hydrolysis at the H^6-P^7 bond (Fig. 2).

From with the information presented in this paper, it can be concluded that the aspartic or acidic proteinases from *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* have similar specificities, including enzyme-substrate affinity and turnover rate.

The pepstatin-insensitive acidic proteinases, A-1 and B, were products of the Deuteromycotina, *Scytalidium lignicolumn*.¹³⁾ The molecular weight of the type A-1 proteinase was found to be 40,000 with $pI=3.6$, the optimum pH being 3.0~3.5. The molecular weight of the type B enzyme was found to be 21,969 with $pI=3.2$, the optimum pH being 2.2. In the present experiments, the type A-1 proteinase exhibited a preference for a hydrophobic amino acid residue at the P_1 position. The type B proteinase was shown here to have high affinity for the H^6-P^7 and H^9-L^{10} bonds in the tetradecapeptide pH 2.2. The type B proteinase exhibited a preference for a hydrophobic amino acid residue at the P_1' position and a hydrophilic amino acid residue at the P_1 position. The two proteinases have different specificities from the others as to tetradecapeptide hydrolysis.

The acidic proteinases from *Cladosporium*, *Rhodotorula glutinis*, *Rhizopus* sp. and *Aspergillus* were inactivated by *Streptomyces*

pepsin inhibitor (S-PI, acetylpepstatin).²⁷⁾ As shown in Fig. 4, the acidic proteinases from two Basidiomycetes, *Rhodotorula glutinis* and *Pycnoporus sanguineus*, and the yeast-like Deuteromycotina, *Cladosporium* sp., could hydrolyze the Y⁴-I⁵ and L¹¹-V¹² bonds, but could not hydrolyze the H⁶-P⁷ bond in the tetradecapeptide, unlike the aspartic and acidic proteinases from *Aspergillus*, *Mucor* and *Rhizopus*.

The present results in Fig. 5 also show that pepsin exhibited a preference for hydrophobic amino acid residues at both the P₁ and P₁' positions, the V³-Y⁴ and L¹⁰-L¹¹ bonds, in the tetradecapeptide. Pepsin additionally cleaved the V¹²-Y¹³ bond at a low reaction rate. The P₃' position may therefore be important for the catalytic efficiency of hydrolysis at the P₁-P₁' bond with pepsin. No synthetic substrate of a defined chemical composition has been found for cathepsin D. Dorer *et al.*²⁸⁾ reported that cathepsin D only cleaved the L¹⁰-L¹¹ bond in the tetradecapeptide, releasing pro-angiotensin (D¹-L¹⁰). In the present study we found a very low hydrolytic activity of cathepsin D towards the Y⁴-I⁵ and F⁸-H⁹ bonds in proangiotensin at pH 3.0. The splitting sites of cathepsin D resembled that of the serine proteinase from *Aspergillus sojae*.²⁹⁾

It can be concluded that the specificities of all aspartic and acidic proteinases of fungal origin are different from those of animal pepsin and cathepsin D.

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