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MOLECULAR AND ENZYMATIC PROPERTIES OF AN ASPARTIC PROTEINASE FROM *RHIZOPUS HANGCHOW*

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Key Word Index—*Rhizopus*; fungus; protease; aspartic proteinase; rhizopuspepsin, specificity.

Abstract—An aspartic proteinase, rhizopuspepsin (EC 3.4.23.21), from *Rhizopus hangchow* was purified. The M_r and isoelectric point were determined as *ca* 37 000 and 4.5, respectively. The first 19 amino acids in the *N*-terminal region were SGSGVVPMTDYEYDIEYYG. The contents of the α-helix, β-structure and random coil were calculated to be *ca* 7.5, 88.9 and 2.7%, respectively. The enzyme can activate trypsinogen at pH 3.0. The activity was completely inactivated by pepstatin A. The specificity and mode of action of the enzyme were investigated with oxidized insulin B-chain at pH 3. The enzyme hydrolysed primarily two peptide bonds, the Leu^{15}–Tyr^{16} bond and the Tyr^{16}–Leu^{17} bond, while additional cleavage of the bonds, Ala^{14}–Leu^{15} and Phe^{24}–Phe^{25} was also noted.

INTRODUCTION

It is well known that aspartic proteinase is widely distributed in various organisms [1]. Previous work in this laboratory led to the characterization and mode of action of the aspartic proteinase, aspergillopepsin I (EC 3.4.23.18), from *Aspergillus saitoi* [2–10]. The M_r value of 34 302 was deduced from data on the nucleotide sequence of the aspergillopepsin I gene, *apnS*, from *Aspergillus saitoi* [10]. Aspergillopepsin I exhibits trypsinogen activation at pH 3 [11, 12]. Of the aspartic proteinases from *Rhizopus chinensis* [13, 14] and *R. niveus* [15], rhizopuspepsins (EC 3.4.23.21) act on various proteins and synthetic substrates. Aspergillopepsin I [11, 12, 16], penicillopepsin (EC 3.4.23.20) [17–22] and rhizopuspepsin [18] exhibit trypsinogen activation at acidic pH.

We have now obtained a purified preparation of an aspartic proteinase, rhizopuspepsin, from *R. hangchow* and here describe its molecular and enzymatic properties. Homology of *N*-terminal peptide sequence of the enzyme was compared with other aspartic proteinases. Specificity of the enzyme for the oxidized B-chain of insulin was recognized.

RESULTS AND DISCUSSION

This purified aspartic proteinase obtained by successive chromatography migrated as a single band on PAGE at pH 7 gel and SDS–PAGE, respectively. Specific activity of the enzyme for casein was 11×10^{-3} kat kg^{-1} protein at pH 3. Yield of the enzyme was *ca* 8%. M_r values of 37 600 and 36 000 were obtained by SDS–PAGE and FPLC with Superose 12, respectively. The isoelectric point was 4.5 by isolectric focusing. The CD spectrum of the enzyme is shown in Fig. 1. The contents of α-helix, β-structure and random coil of the enzyme were calculated to be *ca* 7.5, 88.9 and 2.7%, respectively.

The *N*-terminal peptide sequence of the enzyme was determined as shown in Fig. 2. Homology of the enzyme was compared with other rhizopuspepsins from *R. chinensis* [23, 24] and *R. niveus* [25], and that of *N*-terminal sequence was, respectively, 74 and 68%; sequence homology with other aspartic proteinases was low.

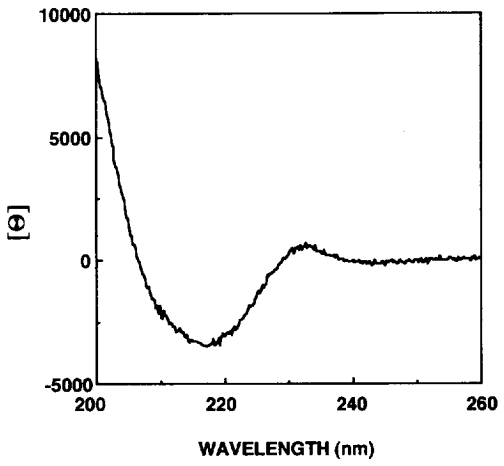

Fig. 1. CD spectrum of the aspartic proteinase from *Rhizopus hangchow* in the region of 200–260 nm.

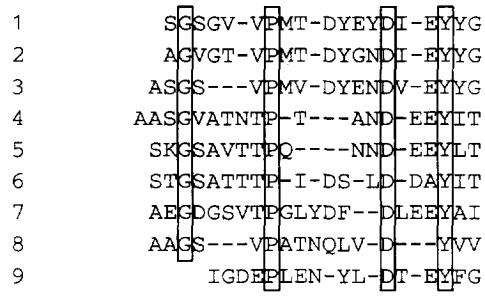

Fig. 2. N-Terminal peptide sesquence determination of the aspartic proteinase from *Rhizopus hangchow* and homology of the enzyme with those of other aspartic proteinases. 1, *Rhizopus hangchow* aspartic proteinase; 2, *R. chinensis* aspartic proteinase [23, 24]; 3, *R. niveus* aspartic proteinase [15]; 4, *Penicillium janthinellum* aspartic proteinase [20, 21]; 5, *Aspergillus saitoi* aspartic proteinase [10]; 6, *Cryphonectoria parasitica* (*Endothia parasitica*) aspartic proteinase [28]; 7, *Rhizomucor pusillus* (*Mucor pusillus*) aspartic proteinase [29]; 8, *Irpex lacteus* aspartic proteinase [30]; 9, porcine pepsin [27].

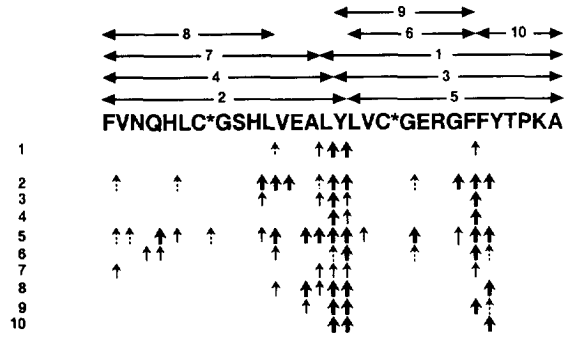

Fig. 3. Summary of the specificity of the aspartic proteinase from *Rhizopus hangchow* and other proteinases towards the oxidized B-chain of insulin at pH 3 and 30°. (A) The enzymatic digest of the chains was directly separated with HPLC. Abbreviations of the amino acids follow the alphabetical system. C* indicates cysteine sulphonic acid. The sign (↔) shows the peptides recovered. Perpendicular arrows (⇑, ↑, ↟) indicate the bond split, the degree of hydrolysis being: ⇑ > ↑ > ↟. (B) 1, Aspartic proteinase from *Rhiozopus hangchow*; 2, *R. chinensis* aspartic proteinase [14]; 3, *Aspergillus saitoi* aspartic proteinase [8]; 4, *A. sojae* aspartic proteinase [34]; 5, *Pencillium janthinellum* aspartic proteinase [19]; 6, *Cryphonectoria parasitica* (*Endothia parasitica*) aspartic proteinase [31]; 7, *Rhizomucor pusillus* (*Mucor pusillus*) aspartic proteinase [35]; 8, human pepsin 3 [36]; 9, human pepsin 5 (36); 10, human non-pepsin proteinase [37].

The optimal pH of the enzyme for trypsinogen activation was 3–4, and the specific activity of the enzyme for this activation was 13.9×10^{-3} kat kg^{-1} protein. The activity was retained up to 45° at pH 3 for 5 min, and the enzyme was completely inactivated at 50°. When the enzyme was incubated at pH 9 and 5° for 18 hr, *ca* 50% of the activity was retained. The activity was completely inactivated by 1 μM pepstatin A and about 56% of the activity was inhibited with *N*-diazoacetylnorleucine-methyl ester (DAN) at 14° for 40 min (enzyme/DAN-/Cu^{2+}; 1:5:100 mol mol^{-1}). Inhibition studies indicate that the enzyme was a member of the pepsin family; it had milk clotting activity (data not shown).

Investigation of the peptide bond specificity towards the B-chain of insulin at pH 3 by the enzyme revealed cleavage of two major bonds, Leu^{15}–Tyr^{16} and Tyr^{16}–Leu^{17}, and two additional bonds, Ala^{14}–Leu^{15} and Phe^{24}–Phe^{25} (Fig. 3).

Twenty-six aspartic proteinases of the pepsin family are listed in the 'Enzyme Nomenclature' [26]. Those are most active at acidic pH and are sensitive to specific inhibitors of pepsin such as pepstatin (hexapeptide), DAN and 1,2-epoxy-3-(-*p*-nitrophenoxypropane) (EPNP) [1]. These aspartic proteinases are all characterized by an M_r of 35 000, and pH optima for catalytic action in the range of 1.5–5.0. The characteristic active site residues are Asp 32 and Asp 215 (porcine pepsin numbering [27]) which are in close proximity to each other. In the enzymatically active pH range of 2–3, Asp 32 is ionized and Asp 215 is unionized. The aspartic proteinases include fungal penicillopepsin (EC 3.4.23.20) [20–22], which serves as a model, mammalian pepsin (EC 3.4.23.1), cathepsin D (EC 3.4.23.5), renin (EC 3.4.23.15), chymosin (EC 3.4.23.4) and certain fungal proteinases, aspergillopepsin I (EC 3.4.23.18), and endothiapepsin (EC 3.4.23.22). The mechanism of activation of bovine trypsinogen by the fungal enzymes, penicillopepsin [17, 18] and aspergillopepsin I [11, 12, 16] is identical with catalytic activation and involves the cleavage of the Lys^6–Ile^7 bond with the liberation of the hexapeptide Val-$(Asp)_4$-Lys.

An acidic proteinase from *R. hangchow* is a member of the aspartic proteinase family. Its homology with *R. chinensis* [23, 24] and *R. niveus* [25] was 74 and 68%, respectively, while it was 37, 37 and 21% with aspergillopepsin I [10], penicillopepsin [20–22] and endothiapepsin [28]; the enzyme had only a low degree of sequence homology with mucorpepsin [29], *Irpex* pepsin [30], endothiapepsin (EC 3.4.23.22) (31) and porcine pepsin [27].

The results in the CD spectrum suggest that the enzyme contains 88.9% β-structure, 7.5% α-helix and 2.7% random coil. Judging by earlier findings on ORD values of rhizopuspepsin from *R. chinensis* [32] and aspergillopepsin I from *A. saitoi* [4, 5], the two fungal enzymes and chymosin [33] contain little or no α-structure. We previously concluded [4, 5] that aspergillopepsin I was devoid of a complete α-helical strand, as judged from the UV ORD curve at the 233 and 198 spectral zone; the conformation of aspergillopepsin I was apparently converted by the anionic detergent, Na-lauryl sulphate (Na-dodecyl sulphate, SDS) into a partially α-helical conformation.

Rhizopuspepsin from *R. chinensis* [14] cleaved the B-chain of oxidized insulin at 12 sites of the peptide linkages and a certain similarity in the specificity was observed among penicillopepsins [19]. The present studies indicate that the sites of the oxidized B-chain of insulin attacked by the enzyme are different from rhizopuspepsin from

R. chinensin [14]. The aspartic proteinase from *R. hangchow* shows a specificity similar to those of aspergillopepsins I from *A. saitoi* [8] and *A. sojae* [34], and to mucorpepsin [35]. In conclusion peptide bonds which have hydrophobic amino acids such as alanine, leucine, tyrosine or phenylalanine in the P_1, P_1' and P_2' positions (as defined by Schechter and Berger [38]) are preferentially cleaved by the enzyme from *R. hangchow*.

EXPERIMENTAL

Materials. Oxidized B-chain of insulin was purchased from Sigma. Butyl-Toyopearl 650M, DEAE-Toyopearl 650S and SP-Toyopearl 650M were from Tosoh Co., Ltd. Folin–Ciocalteu's reagent was from Wako Junyaku Co. Bis-acrylamide was from Nacalai. Pepstatin A and *N*-benzoyl-L-arginine-4-methylcouraryl-7-amide (Bz-Arg-MCA) were from Peptide Institute, Inc., Ina, Minoh-shi, Osaka. Trypsinogen and trypsin were from Sigma. Milk casein (Hammersten) was from Merck.

Enzyme source. A crude enzyme prepn obtained from a commercial product 'Peptidase R' from the *Rhizopus hangchow* No. 3545 of Amano Pharmaceutical Co., Nishikasuga-gun, Aichi Prefecture, was used in this study.

Assay of aspartic proteinase activity. The proteolytic activity with milk casein was used at pH 3 following the previous method [6]. One katal of the aspartic proteinase is defined as the amount of enzyme yielding the colour equivalent of 1 mol tyrosine sec^{-1} with Folin–Ciocalteu's reagent using milk casein as a substrate at pH 3 and 30°. Specific activity is expressed in kat kg^{-1} of protein by the method of ref. [8].

Assay of trypsinogen activation activity. The assay of the enzyme was based on its ability to activate trypsinogen at pH 3. Trypsin formed was assayed at pH 8 according to the method described in ref. [18], except that the substrate, Bz–Arg–MCA, was used. Fluorometric amidase activity of trypsin formed by the free 7-amino-4-methylcoumarin (AMC) in the enzymatic hydrolysate at pH 8 was measured with a Hitachi fluorescence spectrophotometer model F-3000 as in refs [39, 40]. Excitation and emission wavelengths were 360 and 440 nm, respectively.

Protein concn. Protein concns were estimated by the method of ref. [41].

Purification of proteinase. All purification procedures were done at 4°. The crude enzyme soln was dialysed against 10 mM Na-citrate buffer, pH 5.2, with 2 mM $Ca(MeCO_2)_2$ and was centrifuged at 15 000 *g* for 20 min. The supernatant was salted out with 35% $(NH_4)_2SO_4$, centrifuged at 10 000 *g* for 20 min, and applied for hydrophobic chromatography on a Butyl-Toyopearl 650M column (3.7 × 24 cm) equilibrated with the same buffer. The enzyme was eluted with 10 mM Na-citrate buffer, pH 5.2, with a linear gradient $(NH_4)_2SO_4$ from 35 to 0% soln, and the eluate was dialysed against 10 mM citrate buffer, pH 5, with 2 mM $Ca(MeCO_2)_2$. DEAE-Toyoperal 650S (3.7 × 24 cm) was used for the next step in the purification. The enzyme was eluted with the same buffer containing 0.5 M NaCl, and the eluate was dialysed with Na-citrate buffer, pH 3. In the final stage of purification SP-Toyoperal 650M (3.7 × 24 cm) was used. The enzyme was eluted with the same Na-citrate buffer, pH 3, with 0.75 M NaCl.

Polyacrylamide gel electrophoresis (PAGE). Performed using 10% polyacrylamide gel at pH 7 following the method in ref. [42].

Molecular weight. M_r was determined by SDS–PAGE as described in ref. [43]. Marker proteins of cytochrome *c* from horse and cross-linked cytochrome *c* oligomers were monomer (12.4 k), dimer (24.8 k), trimer (37.2 k), tetramer (49.6) and hexamer (74 k); the values in parentheses are the respective M_rs. Markers for gel filtration of FPLC with Superose 12 were β-galactosidase (460 k), amylase (200 k), aldolase (158 k), bovine serum albumin (67 k), ovalbumin (43 k) and soybean trypsin inhibitor (20 k) from Boehringer.

Circular dichroism. CD measurements were made on a JASCO automatic recording spectrophotometer, model J-600, at room temp. (22°) with a 1 mm path length quartz cell from 200 to 260 nm. A circular dichroism scale setting of 0.001 m° cm^{-1} was used in all experiments. Spectra were connected to baseline shifts by a running scale of solvent buffer. Results are expressed as mean residue ellipticities (θ) in deg cm^2 mol^{-1}. The contents of α-helix and β-structure of the aspartic proteinase were calcd according to the SSE-338 Program given in ref. [44].

N-Terminal sequence determination. The enzyme purified by SDS–PAGE was transferred to PVDF membrane, then subjected to N-terminal sequence determination on an Applied Biosystem 473 protein sequencer with 610A data analysis system.

Hydrolysis of oxidized insulin B-chain. The oxidized insulin B-chain (40 nmol) was dissolved in 80 μl of 5 mM Na-citrate buffer, pH 3, and the pH was adjusted to 3, 0.5 pmol of the aspartic proteinase (enzyme/substrate = 1: 80 000, m/m ratio) was added to the soln. The mixt. was incubated at 30° for 5, 20 min or 18 hr. One drop of conc NH_4OH was added to inactivate the enzyme. The sample of hydrolysate was dried *in vacuo*. The dried digests of oxidized insulin-B-chain were dissolved in milli-Q H_2O.

Sepn and identification of peptides. Sepn and identification of the peptides in the dried digests was done by HPLC as described previously [45]. HPLC was performed on a column (4 mm × 25 mm) of ODS-120T (Tosoh) equipped with a Hitachi model L-6200 delivery system. Chromatographic recording was performed at 216 nm with a Shimadzu chromatopac C-RLB.

Peptide analysis. Peptides isolated by HPLC were dissolved in 25 μl 20% acetonitrile and were subjected to sequence determination on an Applied Biosystems 377A protein sequencer.

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REFERENCES

1. Morihara, K. (1974) *Adv. Enzymol.* **41**, 179.
2. Ichishima, E. and Yoshida, F. (1965) *Nature* **207**, 525.
3. Ichishima, E. and Yoshida, F. (1966) *Biochim. Biophys. Acta* **128**, 130.
4. Ichishima, E. and Yoshida, F. (1967) *Agric. Biol. Chem.* **31**, 507.
5. Ichishima, E. and Yoshida, F. (1967) *Biochim. Biophys. Acta* **147**, 341.
6. Ichishima, E. (1970) in *Methods in Enzymology*, Vol. 19 (Perlmann, G. E. and Lorand, L., eds), p. 397. Academic Press, New York.
7. Nakatani, H., Hiromi, K., Satoi, S., Oda, K., Murao, S. and Ichishima, E. (1975) *Biochim. Biophys. Acta* **391**, 415.
8. Tanaka, N., Takeuchi, M. and Ichishima, E. (1988) *Biochim. Biophys. Acta* **485**, 406.
9. Majima, E., Oda, K., Murao, S. and Ichishima, E. (1988) *Agric. Biol. Chem.* **52**, 787.
10. Shintani, T. and Ichishima, E. (1994) *Biochim. Biophys. Acta* **1204**, 257.
11. Gabeloteau, C. and Desnuelle, P. (1960) *Biochim. Biophys. Acta* **42**, 230.
12. Abita, J. P., Delaage, M., Lazdunski, M. and Savrda, J. (1969) *Eur. J. Biochem.* **8**, 314.
13. Fukumoto, J., Tsuru, D. and Yamamoto, T. (1967) *Agric. Biol. Chem.* **31**, 710.
14. Tsuru, D., Hattori, A., Tsuji, H., Yamamoto, T. and Fukumoto, J. (1969) *Agric. Biol. Chem.* **33**, 1419.
15. Kurono, Y., Chidimatsu, M., Horikoshi, K. and Ikeda, Y. (1971) *Agric. Biol. Chem.* **35**, 1668.
16. Nakanishi, K. (1959) *J. Biochem.* **46**, 1553.
17. Northrop, J. H., Kunitz, M. and Herriot, R. M. (1948) in *Crystalline Enzymes*, 2nd Edn, p. 126. Columbia University Press, New York.
18. Sŏdek, J. and Hofmann, T. (1970) in *Methods in Enzymology*, Vol. 19 (Perlmann, G. E. and Lorand, L., eds), p. 372. Academic Press, New York.
19. Mains, G., Takahashi, M., Sodek, J. and Hofmann. T. (1971) *Can. J. Biochem.* **49**, 1134.
20. Hsu, I.-N., Delbaere, L. T. J., James, M. N. and Hofmann, T. (1977) *Nature* **266**, 140.
21. James, M. N. G. and Sielecki, A. R. (1983) *J. Molec. Biol.* **163**, 299.
22. Blundell, T. L., Cooper, J., Foundlimg, S. I., Jones, D. M., Atrash, B. and Szelke, M. (1987) *Biochemistry* **27**, 1140.
23. Delaney, R. R. N. S., Wong, G., Meng, N. W. and Tang, J. (1987) *J. Biol. Chem.* **262**, 1461.
24. Takahashi, K. (1987) *J. Biol. Chem.* **262**, 1468.
25. Horiuchi, H., Yanai, K., Okazaki, T., Takagi, M. and Yano, K. (1988) *J. Bacteriol.* **170**, 272.
26. IUBMB (1992) in *Enzyme Nomenclature* 1992. Academic Press, New York.
27. Tang, J., Sepulveda, P., Marciniszyn, J. Jr, Chen, K. C. S., Huang, W.-Y., Tao, N., Liu, D. and Lanier, J. P. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3437.
28. Barkholt, V. (1987) *Eur. J. Biochem.* **167**, 327.
29. Tonouchi, N., Shoun, H., Uozumi, T. and Beppu, T. (1986) *Nucl. Acids Res.* **41**, 5757.
30. Kobayashi, iH., Sekibata, S., Shibuya, H., Yoshida, S., Kusakabe, I. and Murakami, K. (1989) *Agric. Biol. Chem.* **53**, 1927.
31. Williams, D. S., Whitaker, J. R. and Caldwell, P. V. (1972) *Arch. Biochem. Biophys.* **149**, 52.
32. Tsuru, D., Hattori, A., Tsuji, H. and Fukumoto, J. (1970) *J. Biochem.* **67**, 415.
33. Jirgensons, B. (1965) *J. Biol. Chem.* **240**, 1064.
34. Kimura, T., Mayumi, Y., Takeuchi, M., Hayashi, K. and Ichishima, E. (1979) *Curr. Microbiol.* **3**, 153.
35. Oka, T., Ishino, K., Tsuzuki, H., Morihara, K. and Arima, K. (1973) *Agric. Biol. Chem.* **37**, 1177.
36. Roberts, N. B. and Taylor, W. H. (1979) *Biochem. J.* **179**, 183.
37. Roberts, N. B. and Taylor, W. H. (1978) *Biochem. J.* **169**, 617.
38. Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157.
39. Iida, H., Takeuchi, M. and Ichishima, E. (1988) *Agric. Biol. Chem.* **52**, 1281.
40. Iida, H., Takeuchi, M. and Ichishima, E. (1988) *Agric. Biol. Chem.* **52**, 2313.
41. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
42. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404.
43. Laemmli, U. K. (1970) *Nature* **227**, 680.
44. Yang, J. T. (1986) in *Methods in Enzymology*, Vol. 130 (Jakoby, W. B. and Griffith, O. W., eds), p. 208. Academic Press, New York.
45. Ichishima, E., Takada, Y., Taira, K. and Takeuchi, M. (1986) *Biochim. Biophys. Acta* **869**, 178.