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Grifolisin, a member of the sedolisin family produced by the fungus *Grifola frondosa*

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Abstract

The pepstatin-insensitive carboxyl proteinase grifolisin was purified from fruiting bodies of the fungus *Grifola frondosa*, a maitake mushroom. The enzyme had an optimum pH of 3.0 for the digestion of hemoglobin and 2.8 for milk casein digestion. Its molecular mass was determined to be 43 kDa by SDS–PAGE and 40 kDa by gel chromatography on Superose 12, and its isoelectric point was found to be 4.6 by isoelectric focusing. The enzyme hydrolyzed four major bonds in the oxidized insulin B-chain: Phe1-Val2, Ala14-Leu15, Gly20-Glu21 and Phe24-Phe25 at pH 3.0. The first 15 amino acid residues in the *N*-terminal region were AVPSSCASTITPACL, and the coding region of the grifolisin gene (*gfrF*) has a 1960-base pair cDNA. The predicted mature grifolisin protein consisted of 365 residues and was 26% identical to that of sedolisin from *Pseudomonas* sp. 101 and 34% identical to that of aorsin from *Aspergillus oryzae*. Grifolisin is a member of the sedolisin S53 family and is not inhibited by pepstatin.

Keywords: Grifolisin; Pepstatin; Proteinase; Sedolisin; *Grifola frondosa*

1. Introduction

Fungi are often used to modify foods to make them more nutritious or palatable. In nature, the mushroom *Agaricus bisporus* occurs on mature heaps of garden-waste and along roadsides. The commercial production of *Lentinus edodes* (“SHIITAKE”) was pioneered in Japan, and this mushroom is now widely grown in large quantities; it grows on the dead wood of oaks and related trees. *Grifola frondosa* (“MAITAKE”) is a Basidiomycetes fungus that belongs to the order Aphyllophorales and the family Polyporaceae.

In 1972, using a *Streptomyces* pepsin inhibitor (S-PI, acetyl-pepstatin), Murao et al. (1972) isolated *Scytalidium lignicolum* ATCC 24568, which produces new carboxyl proteinases. The four carboxyl proteinases A-1, A-2, B and C of *S. lignicolum* were insensitive to S-PI and DAN, in contrast to most carboxyl proteinases of the pepsin family. Pepstatin-insensitive carboxyl proteinases have also been found in the fungi *L. edodes* (Terashita et al., 1984b) and *Ganoderma lucidum* (“MANNENTAKE”) (Terashita et al., 1984a). These results suggested that pepstatin-insensitive carboxyl proteinases of *L. edodes* and *G. lucidum* are different from the usual aspartic proteinases of the pepsin family. Sedolisin, a pepstatin-insensitive carboxyl proteinase from *Pseudomonas* sp. 101, has been studied by Oda and coworkers (Oda et al., 1987, 1994; Oyama et al., 1996;

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Ito et al., 1999; Wlodawer et al., 2001). *N*-Isovaleryl-L-tyrosyl-L-leucyl-L-tyrosinal (tyrostatin; Oda et al., 1989) inhibits sedolisin, which is a homolog of tripeptidyl-peptidase I (CLN2 protein) (Rawlings and Barrett, 1999; Lin et al., 2001), a human proteinase associated with a serious neurodegenerative disease. The S53 "sedolisin" family comprises proteinases from bacteria and those from higher organisms. Sedolisins have a unique catalytic triad consisting of Glu80, Asp84 and Ser287 (Wlodawer et al., 2001). This family of sedolisins, S53, is now assigned in MEROPS (http://merops.sanger.ac.uk) as the second member of the SB clan. A review of the structural and enzymatic properties of the sedolisin family of serine-carboxyl peptidases was conducted by Wlodawer et al. (2003).

In this we describe the purification, general properties and substrate specificity against oxidized insulin B-chain of pepstatin-insensitive grifolisin from *G. frondosa*. We also describe the isolation and structural analysis of the cDNA (*gfrF*) coding for grifolisin and compare the deduced amino acid sequence with those of other sedolisins.

2. Results and discussion

During purification, we obtained two carboxyl proteinases from the fruit-bodies of *G. frondosa*. Based on the inhibition experiments with pepstatin, DAN and EPNP, one was a pepstatin-sensitive enzyme and the other was a pepstatin-insensitive enzyme. We focused on the purification, molecular properties, substrate specificity and primary structure of the pepstatin-insensitive caroboxyl proteinase grifolisin. This enzyme was most active toward milk casein at pH 2.8 and toward hemoglobin at pH 3.0, and was stable in the pH range of 1.6–6.0. While the optimal temperature of 40 °C, it lost its activity at 60 °C.

The purified grifolisin obtained by successive chromatographies migrated as a single band during SDS–PAGE, as shown in Fig. 1 and during PAGE in pH 2.3 gel (data not shown), respectively. The purification represented a 47-fold purification over the starting material with about 3.5% recovery. The specific activity of the enzyme toward casein was 8.2×10^{-2} kat kg^{-1} protein. Its molecular mass was estimated to be 43 kDa by SDS–PAGE and 40 kDa by gel chromatography on FPLC with Superose 12. The p*I* value was 4.6 as determined by isoelectric focusing. The secondary structure was estimated from CD measurements. The α-helix, β-structure and random coil contents were calculated to be 43.2%, 28.2% and 28.4%, respectively.

The enzyme was incubated at 4 °C for 30 min with various chemicals in 20 mM citrate buffer at pH 2.8, any remaining activities were then assayed at pH 2.8. While tyrostatin (Oda et al., 1989) and TPCK partly inhibited its activity, it seemed to be unaffected by DAN, EDTA, 1,10-phenanthroline, bestatin, E-64, *N*-ethylmaleimide, iodoacetamide, PCMB, PMSF and chymostatin (Table 1). TPCK might bind due to its peptidyl-ketone moiety and because it lacks a catalytic His residue. Calcium is not required for the activity of this enzyme, since no inhibition was determined in the presence of EDTA around neutral pH. These results suggest that grifolisin differs from aspartic proteinases of the

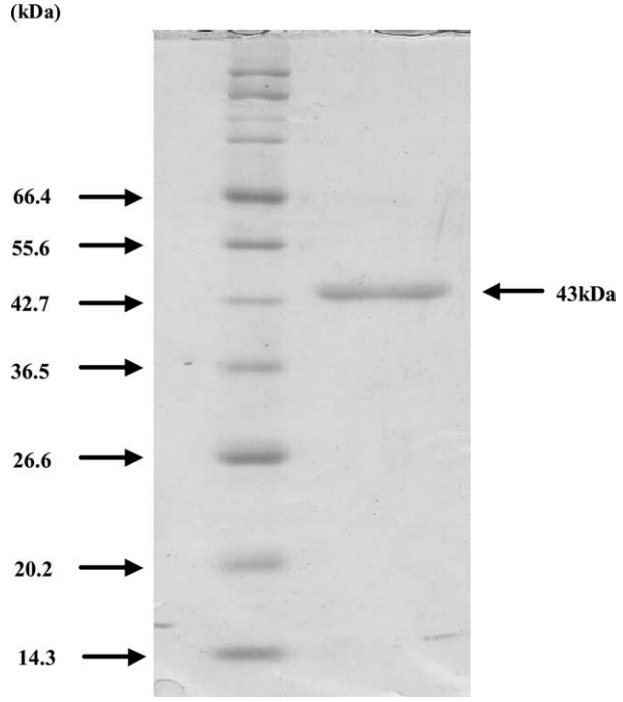

Fig. 1. Analysis of grifolisin from *G. frondosa* by SDS–PAGE. Approx. 1 μg of purified enzyme was separated on SDS–PAGE and stained with Coomasie Brilliant Blue. The left lane represents the molecular mass marker, and the right lane shows a staining band at 43 kDa for grifolisin.

Table 1
Effect of various inhibitors on grifolisin activity

Compound	Concentration (mM)	Relative activity (%)
Control		100
Tyrostatin	0.5	82
Pepstatin	0.1	102
DAN	0.054	100
EPNP	2.23	98
EDTA	10	98
1,10-Phenanthroline	10	101
Bestatin	0.01	97
E-64	0.01	98
NEM	10	98
Iodoacetamide	0.1	92
PCMB	2	95
PMSF	1	99
Chymostatin	0.1	101
TPCK	0.1	86

pepsin family in its catalytic residues and that it is instead a member of the sedolisin family.

The activity of the enzyme toward the oxidized insulin B-chain at pH 3.0 is shown in Fig. 2, where *C** denotes cysteine sulfonic acid. The amino acid sequences of the N-terminal residues from the major products obtained with grifolisin were determined to be VNH, LYL, EGR and FYT, respectively. Grifolisin hydrolyzed four major bonds: Phe1-Val2, Ala14-Leu15, Gly20-Glu21 and Phe24-Phe25. The N-terminal sequences of the minor products were HLV, VEA, YLV, RGF and YTP, respectively. Five minor cleavages occurred at Ser9-His10, Leu11-Val12, Leu15-Tyr16, Glu21-Arg22 and Phe25-Tyr26. According to these cleavage sites the enzyme seems to have quite broad specificity. However, the results indicate that grifolisin tends to prefer aromatic and hydrophobic amino acid residues at the P1–Pi′ position. Schechter and Berger (1967) showed that the active site of papain can be considered to consist of seven subsites (S1–S4 and S1′–S3′), each of which accommodate one amino acid residue of a substrate (P1–P4 and P1′–P3′) located at the N-terminal and C-terminal cleavage sites, respectively. These results were summarized and compared with those of other proteinases of the sedolisin family from Basidiomycetes: *L. edodes* (Terashita et al., 1984b) and *G. lucidum* (Terashita et al., 1984b) (Fig. 2). These three fungal sedolisins all cleaved Leu15-Tyr16 and Phe24-Phe25 bonds. Since only limited specificity data are available for grifolisin, we cannot get any conjecture on appearance of the subsites (S2, S1 and S1′) and thus the observed specificity.

Six peptides, AVPSSCASTITPACL, TGWSPVTGLGXXNFAK, LAVSGYIEQFANQADLK, FVPTFPXGXPFM, FNTSGRGFPDVSTQGENFQIVXDGQXGXVD and SPLGFLNPFLYSTGASAFNSITSGXNPGCNT were obtained from lysylendopeptidase hydrolysis of the enzyme from *G. frondosa*.

We determined the nucleotide sequence of the 1960-bp cDNA (*gfrF*) encoding grifolisin and the coding nucleotide sequence for its known amino acid sequence (Fig. 3). We determined the position of the initiation codon of methionine, ATG, which is the signal for the initiation of protein synthesis. The six underlined sequences in Fig. 3 correspond to the sequences found in the analysis of native grifolisin. The N-terminal amino acid sequence of the mature enzyme was found at positions Ala236 to Leu250. Consequently, the pre-propeptide consisted of 235 amino acid residues from Met1 to Leu235, and the mature form of grifolisin consisted of 365 amino acid residues. The molecular mass of the putative mature protein was calculated to be 43 kDa, which is similar to the value obtained in the SDS–PAGE experiment. The deduced amino acid sequence of the propeptide contained two sites indicated by circles for potential asparagine-linked glycosylation at positions 231 and 487 (252, mature grifolisin numbering), as shown in Fig. 3.

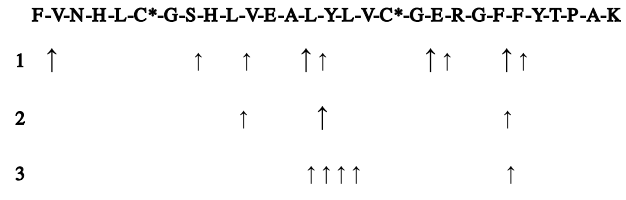

Fig. 2. Specificities of three pepstatin-insensitive carboxyl proteinases (1–3) toward the oxidized insulin B-chain, where *C** denotes cysteine sulfonic acid. 1, Grifolisin from *Grifola frondosa*. 2, Pepstatin-insensitive carboxyl proteinase from *Lentinus edodes* (Terashita et al., 1984b). 3, Pepstatin-insensitive carboxyl proteinase from *Ganoderma lucidum* (Terashita et al., 1984a). ↑, Major cleavage site. ↑, Minor cleavage site.

Sedolisins are proteolytic enzymes in which folding resembles that in subtilisin. However, they are considerably larger: their mature catalytic domains contain approximately 375 amino acids (Wlodawer et al., 2003). The defining features of these enzymes are a unique catalytic triad, Ser-Glu-Asp, as well as the presence of an aspartic acid residue in the oxyanion hole (Wlodawer et al., 2001). High-resolution crystal structures have been solved for sedolisin from *Pseudomonas* sp. 101, as well as for kumamolisin from the thermophilic bacterium *Bacillus* novosp. Mn-32 (Comellas-Bigler et al., 2002) and kumamolisin-As (initially named ScpA) from the thermoacidophilic bacterium *Alicyclobacillus sendaiensis* NTAP-a (Wlodawer et al., 2004). The availability of these crystal structures enabled us to model the structure of mammalian CLN2, an enzyme which, when mutated in humans, leads to a fatal neurodegenerative disease (Sleat et al., 1997).

A prominent octahedrally-coordinated Ca^{2+}-binding site of sedolisin is liganded by two carbohydrate oxygens of Asp328 and Asp348, three amide carbonyl groups of residues 329, 344 and 345, and a clearly defined water molecule (Wlodawer et al., 2001). The sequence alignment of mammalian and fungal enzymes reveals that Asp328 and Asp348 on the C-terminal region are conserved in all members of the sedolisin family, including sedolisin, TPP-I, physarolisin, kumamolisin and kumamolisin-B. A previous study also determined that the amino acid residues Asp395 and Asp416 are conserved in aorsin from *Aspergillus oryzae*, which are equivalent to Asp328 and Asp348 in sedolisin, respectively (Lee et al., 2003). Aorsin contains 1 mol of calcium per mol of enzyme.

The amino acid sequences of the sedolisin family are compared in Fig. 4. The deduced amino acid sequence of grifolisin showed several conserved amino acid residues, Glu85, Asp89, Asp175 and Ser283, which are equivalent to Glu80, Asp84, Asp170 and Ser287 in sedolisin, respectively, and all of which have been previously described as being essential for enzyme catalytic activity (Ito et al., 1999; Oyama et al., 1996).

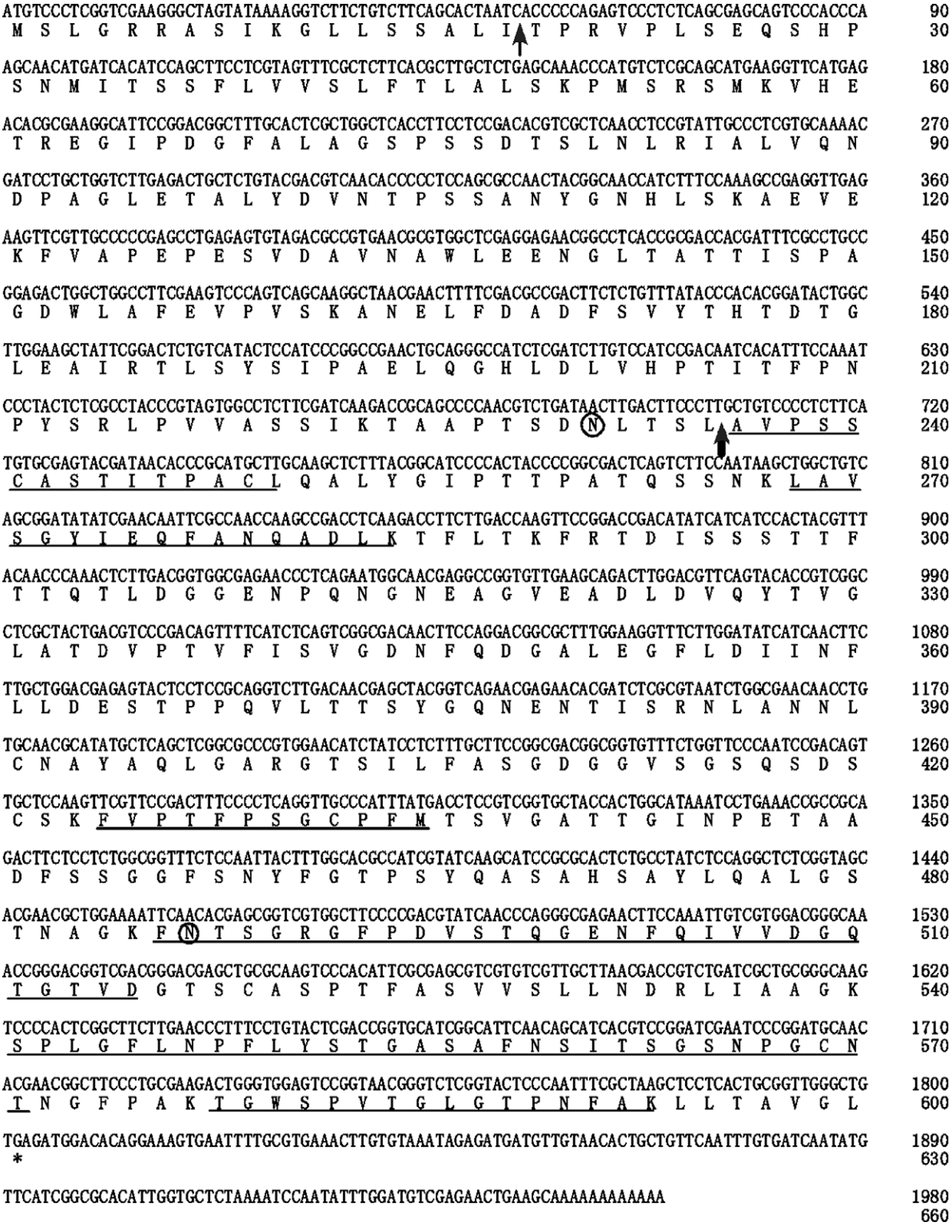

Fig. 3. Nucleotide and deduced amino acid sequences of the grifolisin gene. The predicted amino acid sequence of grifolisin from *G. frondosa* is shown below the cDNA sequence. Standard single-letter symbols are used. The N-terminal amino acid sequence of purified grifolisin and the internal amino acid sequences isolated after lysylendopeptidase digestion are underlined. The thin arrow (↑) marks the putative cleavage site of signal peptide according to the weight–matrix approach of von Heijne (1986), and the broad arrow (↑) indicates the start of the mature enzyme. Circles indicate the N positions of potential N-linked glycosylation sites.

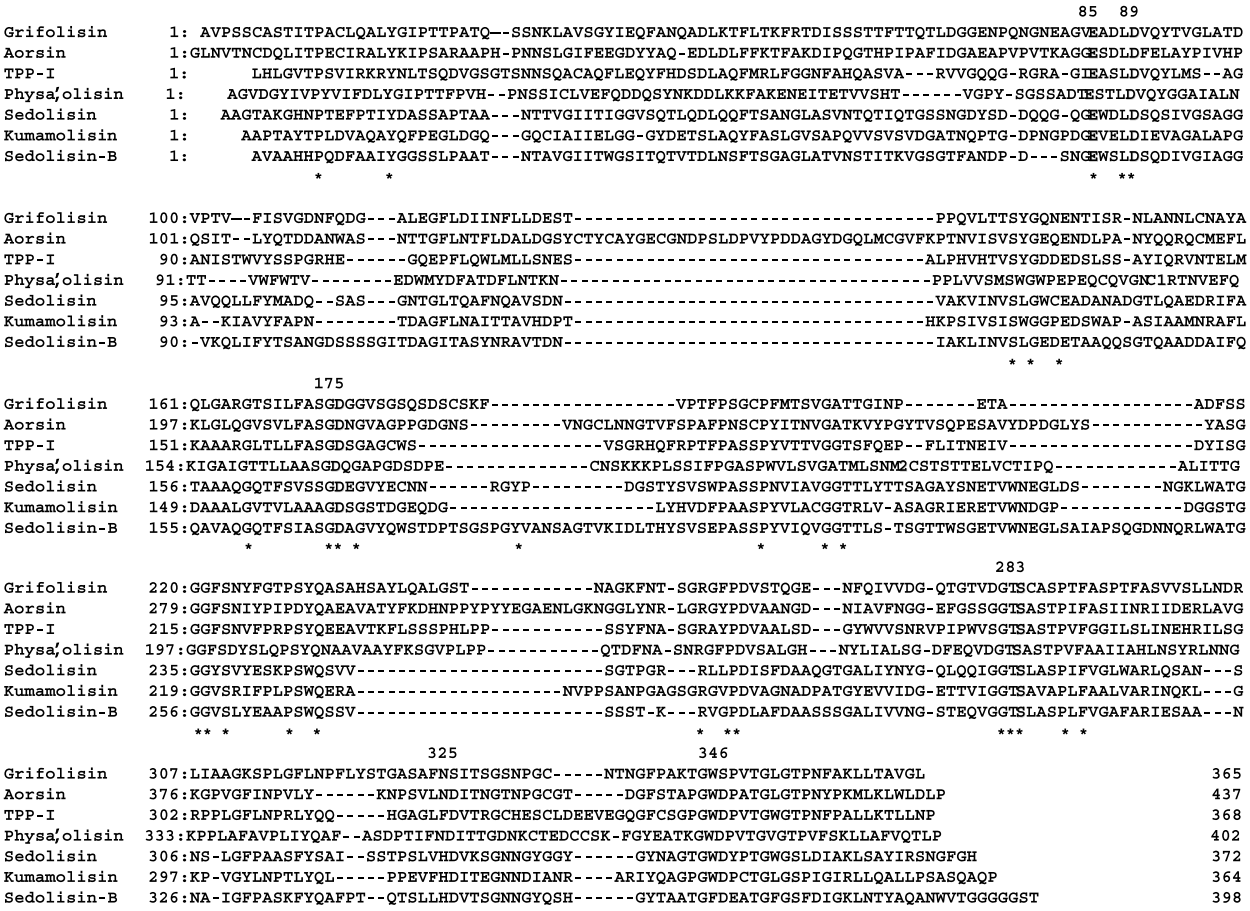

Fig. 4. Comparison of the amino acid sequences of pepstatin-insensitive carboxyl proteinases (serine-carboxyl proteinases). The amino acid sequence of grifolisin is compared with those of aorsin from *A. oryzae* (Lee et al., 2003), human tripeptidyl-peptidase I (TPP-I, CLN2) (Rawlings and Barrett, 1999; Lin et al., 2001), physarolisin (Nishii et al., 2003) (formerly called phasaropepsin, from the slime mold (mycetozoa) *Physarum polycephalum*), sedolisin (PSCP) (Oda et al., 1987, 1994; Oyama et al., 1996; Ito et al., 1999; Wlodawer et al., 2001), kumamolisin (Murao et al., 1993; Comellas-Bigler et al., 2002) from the thermophilic bacterium *Bacillus* novo sp. MN-32 and sedolisin-B (Oda et al., 1996) from the bacterium *Xanthomonas* sp. T-22. Gaps (−) were introduced to obtain maximum alignment. The numbering above the sequences is that of grifolisin. Amino acid residues that are the same in grifolisin and other sedolisins are indicated by asterisks (*).

3. Conclusions

Grifolisin from *G. frondosa* is a member of the S53 family of serine-carboxyl peptidases (Wlodawer et al., 2003), or sedolisins, and is not inhibited by pepstatin. Grifolisin appears to differ from all other known members of the sedolisin family by an apparent lack of the universally conserved Ca^{2+}-binding sites. The deduced amino acid sequence of grifolisin showed the presence of amino acid residues Ser325 and Ser346, which are equivalent to Asp328 and Asp348 in sedolisin, respectively.

4. Experimental

4.1. Materials

Commercially available fruiting bodies of *G. frondosa* were supplied by Yukiguni-Maitake Co., Ltd., (Niigata) and stored at −20 °C until use. The mushrooms had been harvested from cultures grown on a mixture of hardwood sawdust and corn bran (3–1, w/w) at 25 °C for 90 days. A pepstatin-insensitive acidic proteinase from *G. frondosa* was extracted with 50 mM acetate buffer pH 5.0 from crushed fruiting bodies after the large-scale commercial growth of *G. frondosa* as described below. Lysylendopeptidase and the oxidized B-chain of insulin were purchased from Sigma (St. Louis, MO). SP-TOYOPEARL, Super-Q-TOYOPEARL and RESOURCE Q were acquired from TOSOH Co., Ltd. (Tokyo). Pepstatin A, *N*-[*N*-(L-3-transcarboxyoxirane-2-carbonyl)-L-leucyl] agmatine (E-64), bestatin and chymostatin were purchased from Peptide Institute Inc. (Mino-city, Osaka), and diazoacetyl-DL-norleucine methyl ester (DAN), *N*-ethylmaleimide (NEM) and 1,10-phenanthroline were purchased from Sigma (St. Louis, MO). *N*-tosyl-L-phenylalanyl chloromethane (TPCK), Folin–Ciocalteau's reagent, iodoacetamide,

p-chloromercuribenzoate (PCMB) and chymostatin were obtained from Nacalai Tesque (Osaka), 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (EPNP) was obtained from Kanto Chemical Co., Ltd. (Tokyo) and milk casein (Hammersten) was acquired from Merck. All other chemicals were of reagent grade. Tyrostatin was a kind gift from Prof. K. Oda of the Kyoto Institute of Technology.

Escherichia coli DH5α [*supE*44 Δ*lacU*169(φ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] was used for plasmid isolation and cloning.

4.2. Enzyme assay

Proteolytic activity was assayed at pH 2.8 with milk casein according to a previous paper (Ichishima, 2004). One katal (kat) under the SI system (Price and Stevens, 1999) of enzyme activity is the amount of enzyme that yields the color equivalent of 1 mol tyrosine per second with Folin–Ciocalteau's reagent using milk casein as a substrate at pH 2.8 and 30 °C. Specific activity was expressed in kat per kg protein.

4.3. Effects of inhibitors on grifolisin activity

A mixture containing 0.5 ml of 20 mM sodium acetate buffer (pH 5.0), 0.1 ml inhibitor solution (in the presence of 0.23 mM $CuSO_4$ for DAN), and 0.1 ml of enzyme solution in a test tube was incubated at 4 °C for 24 h (72 h for DAN and EPNP); the enzyme reaction was then initiated by adding 0.5 ml 1.0% casein as a substrate. The subsequent procedure was the same as that described in the section on the assay of the enzyme activity. Control runs (100% activity) were prepared by replacing the inhibitor solutions with distilled water. The following components were used: tyrostatin, pepstatin, DAN, EPNP, EDTA, 1,10-phenanthroline, bestatin, E-64, NEM, iodoacetamide, PCMB, PMSF, chymostatin and TPCK.

4.4. Enzyme purification

Frozen fruiting bodies (1 kg) were dissected into small pieces and homogenized with 1 l of 50 mM sodium acetate buffer pH 5.0 in a blender for 20 min at 4 °C. The homogenate was centrifuged at 10,800 *g* for 15 min at 4 °C. The supernatant was then brought to 30% ammonium sulfate saturation, and the resulting precipitate was centrifuged at 10,800 *g* for 30 min and discarded. The resulting supernatant was next brought to 80% saturation with ammonium sulfate and left to stand for 16 h at 4 °C. After centrifugation at 12,200 *g* for 30 min, the precipitate was dissolved in 80 ml of 10 mM citrate buffer pH 3.0. The solution was dialyzed against the same buffer for 2 days at 4 °C. The dialyzed solution was then centrifuged at 22,200 *g* for 15 min and the supernatant was applied to a SP-TOYOPEARL column (3 × 8 cm) that had been previously equilibrated with 10 mM citrate buffer pH 3.0. Elution was performed with a linear gradient of 0–0.3 M sodium chloride in 10 mM citrate buffer pH 3.0. The active fractions were dialyzed overnight against 20 mM sodium acetate buffer pH 5.0 at 4 °C and the dialyzed solution was applied to a Super Q-TOYOPEARL column (3 × 8 cm) that had been equilibrated with 20 mM sodium acetate buffer pH 5.0. Elution was conducted with a linear gradient of 0–0.4 M sodium chloride in 20 mM sodium acetate buffer pH 5.0. The active fractions were then dialyzed overnight against 20 mM sodium acetate buffer pH 5.0 at 4 °C and applied to RESOURCE Q (1 ml) that had been equilibrated with the same buffer. Elution was carried out with a linear gradient of 0–0.1 M sodium chloride in 20 mM sodium acetate buffer pH 5.0. The active fractions were finally dialyzed against 20 mM sodium acetate buffer pH 5.0.

4.5. Protein concentration

Protein concentrations were usually estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

4.6. PAGE and SDS–PAGE

PAGE was carried out using 7.5% polyacrylamide gel at pH 2.3 following the method of Davis (1964). SDS–PAGE was performed using the method of Laemmli (1970). Gels were fixed and stained for 8 h in 0.25% Coomassie Brilliant Blue R-250, 50% methanol, and 10% glacial acetic acid solution and then destained with 40% methanol–70% acetic acid solution.

4.7. Activity toward the oxidized insulin B-chain

Reaction mixtures composed of 10 nmol oxidized insulin B-chain were dissolved in 180 μl of 50 mM citrate buffer pH 3.0, and the pH was adjusted to 3 by adding 50 pmol of the enzyme substrate mixture (substrate:enzyme = 200:1, mol:mol ratio) to the solution. The reaction mixture was incubated at 30 °C for 1 and 24 h and then immediately lyophilized.

4.8. Molecular mass determination

Molecular mass values were determined by SDS–PAGE as described in Laemmli (1970) and gel filtration column chromatography of FPLC was performed with Superose 12.

4.9. Isoelectric point (pI) determination

The p*I* value of grifolisin was determined by isoelectric focusing using a Model 11 Mini IEF Cell (BIO RAD).

4.10. N-terminal sequence determination

The enzyme purified by SDS–PAGE was transferred to a polyvinylidene difluoride (PVDF) membrane and subjected to *N*-terminal sequence determination on an Applied Biosystems 473 protein sequencer with a 610A data analysis system.

4.11. Circular dichroism (CD)

CD measurements were made on a JASCO J-720 spectrophotometer at room temperature at 22 °C with a 1 mm path-length quartz cell from 190 to 250 nm. Spectra were connected to baseline shifts by a running scale of solvent buffer. The results were expressed as the mean residue ellipticity (θ) in deg cm^2 mol^{-1}. The α-helix and β-structure content of the enzyme were calculated using the CONTIN circular dichroism program (CPC Program Library, Queen's University of Belfast, Ireland N.).

4.12. Partial amino acid sequencing

Grifolisin was pyridylethylated according to the method of Friedman et al. (1970). Pyridylethylated grifolisin was digested with lysylendopeptidase at 4 M urea and pH 8.9. The peptide fragments were then separated by HPLC using an LCSS-905 HPLC System Station (JASCO) and an AQUA C18 column (Phenomenex) with a linear gradient of acetonitrile from 10% to 90% containing 0.1% TFA in 30 ml. The flow rate was 1 ml per min and detection was performed at 220 nm. The *N*-terminal amino acid sequences of the peptide fragments were analyzed using an Applied Biosystem 473A protein sequencer.

4.13. cDNA cloning

The oligo-nucleotide primers Fr6 (5′-GCIAGIACS ATHACNCCNGC-3′) and Fr9 (5′-ACIATYTGRA ARTTYTCNCCYTG-3′) were synthesized, based on the *N*-terminal amino acid sequence (-ASTITPI-) and internal sequence (-QGENFQIV-), respectively. Total RNA from fruiting bodies of *G. frondosa* was isolated using an RNeasy Mini Kit (QIAGEN). A 1.2-kb DNA fragment was amplified by genomic PCR with primers Fr6 and Fr9 according to the methods of Loh et al. (1989) and Ohara et al. (1989) and the nucleotide sequence was then determined. The specific primers NF1 (5′-CTGGCTGTCAGCGGATATATCGA-3′) and NFE (5′-TTCGCCAACCAAGCCGACCTCAA G-3′) were synthesized based on this DNA sequence. First-strand cDNA was synthesized from total RNA using the oligo dT-anchor primer (5′-GACCACG CGTATCGATGTCGACTTTTTTTTTTTTTTT-3′), AMV reverse transcriptase and the deoxynucleotide mixture. Full-length cDNA was obtained by the RACE (rapid amplification of cDNA ends) method using a 5′/3′ RACE Kit (Roche). The specific primers NF1 and NFE and anchor primer (5′-GACCACGCGTATCGATGT CGAC-3′) were used for 3′-side cDNA amplification.

Finally, a full-size 1.4-kb cDNA fragment was obtained by RT-PCR using the sense primer SqN2 (5′-GAGCTAACAAACATGAA-3′) and the antisense primer Sq-R35 (5′-AGGCTGCAGAAGCTTTCCTGC ATTTACTTGGCAAC-3′). Cloned cDNA was subcloned into pBluescript II and the nucleotide sequence was determined using an SQ-5500 DNA sequencer (HITACHI).

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