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Phytochemistry 66 (2005) 983–990
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

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

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Received 3 August 2004; received in revised form 27 December 2004
Available online 26 April 2005

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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doi:10.1016/j.phytochem.2005.02.014

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

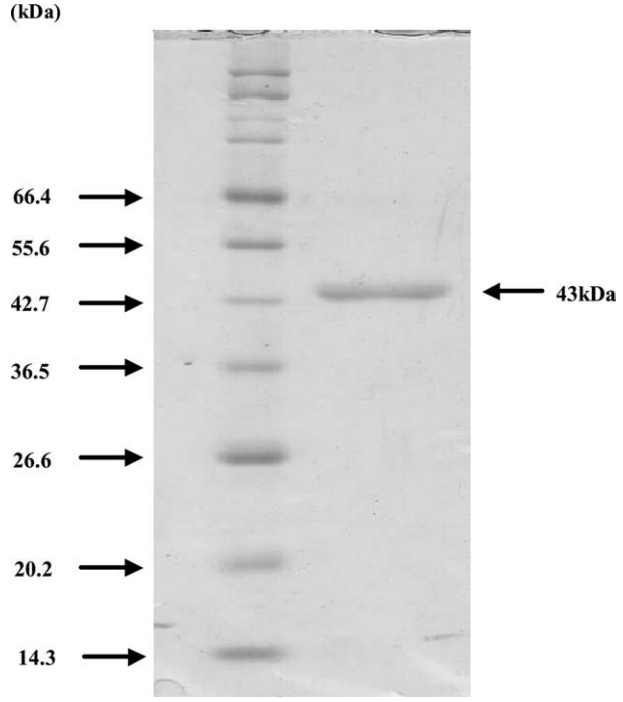

Fig. 1. Analysis of grifolisin from *G. frondosa* by SDS–PAGE. Approx. 1 μg of purified enzyme was separated on SDS–PAGE and stained with Coomasie Brilliant Blue. The left lane represents the molecular mass marker, and the right lane shows a staining band at 43 kDa for grifolisin.

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