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FULL PAPER

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The stimulation of extracellular carbohydrases of edible mushrooms by the hot water-soluble fraction from corn fiber

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Abstract Corn fiber (CNF) is an abundant by-product of the wet corn milling process in the production of cornstarch. We have shown that the hot water-soluble fraction (HWSF) from CNF has a promoting effect on the mycelial growth of various edible mushrooms, including mycorrhizal fungi. To reveal the promoting mechanisms, the effect of CNF-HWSF on the stimulation of extracellular enzymes was examined. The production of extracellular carbohydrases such as amylase, CMCase, and xylanase was markedly enhanced by the addition of low molecular weight fractions (less than MW 500) prepared from CNF-HWSF. The enzymatic stimulation and enhancement of mycelial growth appeared during 3–15 days after inoculation. Furthermore, a fraction of less than MW 500 was separated by gel filtrate chromatography into five fractions (A–E), and the effect of each fraction was investigated. Promoting effects were shown from C and D fractions; mycelial growth and enzyme production of *Lentinula edodes* were indicated although fraction D has no sugars and amino acids in CNF-HWSF. From these results, the promoting effect of CNF-HWSF seems to be a two-step reaction. The first step could be achieved by rich nutrients such as free amino acids and monosaccharides from CNF-HWSF. The second step (during 3–15 days) is considered to be that the marked promoting effect was caused by the stimulation of extracellular enzymes.

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Introduction

Although abundant corn fiber (CNF) is discharged as a by-product of the wet corn milling process of the production of cornstarch in factories, CNF has hardly any practical use. We attempted to make use of CNF for sawdust-based cultivation of the mushroom, and have previously reported CNF is effective in producing the fruit-body of edible mushrooms, which increases yield, shortens the cultivation period, and increases the quality of mushrooms (Arai et al. 2003a). Also, we described that the hot water-soluble fraction (HWSF) from CNF has a promoting effect on the mycelial growth of various edible mushrooms (1.4–9.5 times that of the control) by adding 5%–20% CNF-HWSF to the medium (Arai et al. 2002, 2003b). These promoting effects were also apparent on mycorrhizal mushrooms, such as *Tricholoma matsutake* (3.3-fold) and *Lyophyllum shimeji* (3.7-fold).

The promoting effects on mycelial growth were shown on media containing the low molecular weight fraction (MW <500 daltons) prepared from CNF-HWSF. Furthermore, the promotive actions were more effective on slow-growing mushrooms (such as *Lentinula edodes* and *Pholiota nameko*) than on rapidly growing mushrooms (such as *Pleurotus ostreatus* and *Flammulina velutipes*).

In this article, to reveal the promoting mechanisms on the growth of mushrooms with CNF-HWSF, we examined the effect of CNF-HWSF components on the stimulation of extracellular enzymes such as amylase, CMCase, and xylanase.

Materials and methods

Strains

Lentinula edodes (Mori no. 465), *Hypsizygus marmoreus* (Takara no. 1), *Pleurotus ostreatus* (Kitamura; obtained from Kin-ki Nyugyo, Japan), and *Flammulina velutipes* (IFO 7777) were used in this study. As inocula, a mycelial block was cut from a plate culture that had grown on a potato dextrose agar (PDA) medium (Nissui, Japan) for 14 days at 24°C in a Petri dish (diameter, 90 mm).

Preparation of CNF-HWSF

One hundred grams CNF was mixed with 1 l distilled water and extracted for 3 h at 80°C. After the residue was removed by centrifugation at 10000 *g* at 0°C for 10 min, the supernatant solution was concentrated at 40°C to 100 ml by a rotary evaporator.

Fractionation and isolation of CNF-HWSF

Ethanol, 400 ml, was added to 100 ml CNF-HWSF and left overnight at room temperature. The sample was separated by centrifugation (0°C, 15 min at 13000 *g*) into the supernatant and the precipitate. The supernatant was further separated into MW 500 or less (500-L) and more (500-M) fractions by molecular sieving using ultrafiltration membranes (YC 05; Amicon, Japan).

Arabinoxylan from the CNF-HWSF was isolated according to Takeuchi (1997) by using 3.5 g freeze-dried CNF-HWSF (this weight equivalent to 100 ml CNF-HWSF). The preparation of cellulose and starch in CNF-HWSF was performed as reported previously (Arai et al. 2003b).

Medium compositions, inoculations, and culture conditions

The potato dextrose liquid (PDL) medium consists of potato extract (200 g boiled in 500 ml distilled water), 15 g glucose, and 1 mg thiamine hydrochloride per liter of distilled water. The PDL medium was supplemented with CNF-HWSF, and each fraction from CNF-HWSF solution of the same concentration as that of CNF-HWSF was dispensed in 16-ml aliquots in 100-ml Erlenmeyer flasks, before autoclaving at 121°C for 10 min. As inoculum, a mycelial block (diameter, 5 mm) was cut from a plate culture. The incubation was carried out at 24°C for 15 days.

Enzyme assays

After separation of mycelium by filtration, the culture filtrate was assayed for enzyme activities. The culture filtrate was dialyzed with 25 mM McIlvaine buffer at each optimum pH and used as the crude enzyme. Amylase, cellulase, and xylanase activities were determined by measuring the amount of glucose or xylose released from soluble starch, carboxymethylcellulose (CMC), and xylan by the Somogyi–Nelson method (Somogyi 1952) with glucose or xylose as the standard. Reaction mixtures contained 90 µl 100 mM McIlvaine buffer at each optimum pH, 90 µl 0.5% (wt/vol) soluble starch, CMC, and xylan solution, and 20 µl crude enzyme solution. After incubation at 37°C for 60 min, the reaction was terminated by adding 200 µl Somogyi reagent. The mixture was vortexed, placed in a boiling-water bath for 10 min, and cooled on ice, and then 200 µl Nelson reagent was added. After being vortexed, the mixture was allowed to stand at room temperature for 20 min and centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 655 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of each reducing sugar per minute.

Organic solvent fractionation

The 500-L fraction was extracted two times with hexane of twice the volume of the 500-L solution. The mixture was separated into the hexane layer and water layer. Then, the water layer was extracted successively with benzene, diethyl ether, dichloromethane, and chloroform, and the respective organic layers and water layer were obtained.

Gel filtrate chromatography

One milliliter of the water layer, which concentrated the quantity 20 fold, was applied to a Sephadex G-10 (Amersham Pharmacia) column (1.5 × 100 cm) equilibrated with distilled water. The column was eluted by distilled water at a flow rate of 1 ml/min and fractions of 3 ml were collected. For the obtained fractions, qualitative analysis of sugars and amino acids followed the Somogyi–Nelson method, high pressure liquid chromatography (HPLC), and ninhydrin reaction, and was separated into five fractions for assay as follows: 1–20, containing a few oligosaccharides (A); 21–24, containing oligosaccharides (B); 25–34, containing monosaccharides and amino acids (C); 35–60, containing no sugar or amino acids (D); 60–80, containing no sugar and amino acids (E).

Measurement of dry mycelial weight

The vegetative mycelia after incubation were separated from the medium by filtration and washed thoroughly with distilled water, after which it was then dried at 80°C for 24 h. The dry weight was measured after cooling in a desiccator.

Results

Effect of CNF-HWSF on extracellular enzymes production of mushrooms

To search for the promoting mechanism on the growth of mushroom fungi by the addition of a CNF component in the culture medium, we examined the effect of extracellular enzyme productions from *L. edodes* (Table 1). Three kinds of carbohydrase activities derived from *L. edodes* were increased by the addition of CNF-HWSF; the production of enzymes rose to a maximum at 20% CNF-HWSF. We have described that the mycelial growth of *L. edodes* was activated with CNF-HWSF and peaked at addition of 20% sample (Arai et al. 2003b); extracellular enzyme production by CNF-HWSF seem to correlate with growth promotion of mycelium. Also, the stimulation of extracellular enzyme productions by CNF-HWSF is found with other edible mushrooms such as *H. marmoreus*, *P. ostreatus*, and *F. velutipes* (Table 2). Three kinds of carbohydrase activities were obviously increased by the addition of CNF-HWSF compared to the control. From these results, it was suggested that enzymatic stimulation with the CNF-HWSF is a common phenomenon in mushrooms.

Effect of fractions from CNF-HWSF on the extracellular enzyme production of *L. edodes*

Each fraction obtained from ethanol treatment and ultrafiltration was added to the PDL medium of *L. edodes*, and the extracellular carbohydrase (amylase, CMCase, and xylanase) production was measured at 15 days after inoculation (Fig. 1). Obviously high values resulted when the enzyme production of the medium, on which fungi had been cultivated on a PDL medium, included low molecular weight fractions such as supernatant of ethanol treatment (C) and the fraction of less than MW 500 (E) in CNF-HWSF. The results from *H. marmoreus*, *P. ostreatus*, and *F. velutipes* also indicated a tendency similar to that of *L. edodes* (data not shown). There was no effect on either mycelial growth or exocarbohydrase production when the chemical reagents glucose, xylose, arabinose, or polysaccharides such as xylan and cellulose were added into the medium.

Time course of vegetative mycelial growth and the xylanase production of *L. edodes*

In general, mushroom fungi have many strains that have high xylanase activity during the growth. We investigated

Table 1. Effect of CNF-HWSF on extracellular enzyme productions from *Lentinula edodes*

Supplement concentration (%)	Enzyme activities (mU/ml)		
	Amylase	CMCase	Xylanase
0	8.51	1.14	0.38
10	55.34	14.77	6.79
20	90.10	34.42	17.47
30	44.90	11.90	3.65

CNF-HWSF, hot water-soluble fraction of corn fiber
Cultures were grown in 100-ml Erlenmeyer flasks containing 16 ml potato dextrose liquid (PDL) medium at 24°C for 15 days
Carbohydrase activities were measured by Somogyi–Nelson method in 0.1 M Mcllvaine buffer pH 4.0 at 37°C

Table 2. Effect of CNF-HWSF on extracellular enzyme productions from edible mushrooms

CNF-HWSF (%)	Enzyme activities (mU/ml)		
	Amylase	CMCase	Xylanase
Hypsizygus marmoreus			
0	1.08	n.d.	1.60
10	4.08	2.33	4.72
Pleurotus ostreatus			
0	34.31	1.10	1.68
20	55.44	2.94	5.29
Flammulina velutipes			
0	18.62	2.15	2.27
20	45.66	10.74	7.58

Cultures were grown in 100-ml Erlenmeyer flasks containing 16 ml PDL medium at 24°C for 15 days
Carbohydrase activities were measured by Somogyi–Nelson method in 0.1 M Mcllvaine buffer (*H. marmoreus*, pH 6.0; *P. ostreatus* and *F. velutipes*, pH 5.0) at 37°C
n.d., not detected

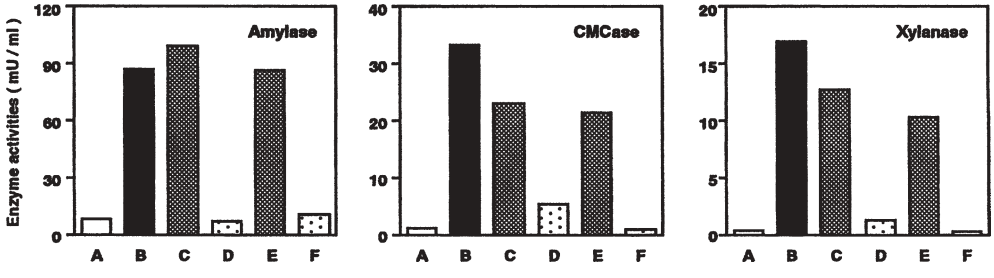

Fig. 1. The influence of hot water-soluble fraction of corn fiber (CNF-HWSF) components on the extracellular enzyme production of *Lentinula edodes*. The vegetative mycelia were cultured for 15 days at 24°C in potato dextrose liquid (PDL) medium with added CNF-HWSF (20%). *A*, Control; *B*, CNF-HWSF; *C*, supernatant of ethanol treatment; *D*, precipitate of ethanol treatment; *E*, 500-L by ultrafiltration; *F*, 500-M by ultrafiltration

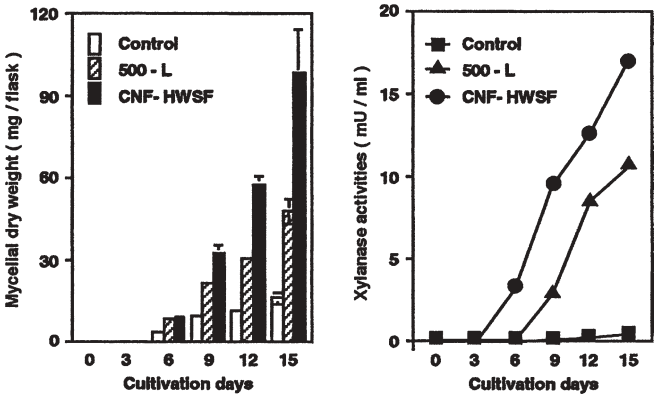

Fig. 2. Time course of vegetative mycelial growth and xylanase production in the PDL medium for *L. edodes*. Vegetative mycelia were cultured at 24°C in a PDL medium with added CNF-HWSF (20%). *Error bars* indicate standard deviation (SD) ($n = 6$)

the relationship between the mycelial growth and the xylanase production in the culture filtrate of *L. edodes*, which is the most important and popular edible mushroom in Japan.

Figure 2 shows the time course of mycelial growth and xylanase production in the medium during the growth of *L. edodes* colonies. When the CNF-HWSF or 500-L fraction was added in the culture medium, the mycelial growth was enhanced from the initial stage of the cultivation period. In particular, there was a great difference in mycelial dry weight with the addition of CNF-HWSF components during 6–15 days after inoculation. Furthermore, only the mycelial growth with CNF-HWSF observed a remarkable increase at the end of the incubation period (12–15 days).

On the other hand, xylanase activity was detected at 3–6 days after inoculation on the CNF-HWSF components supplemented. This activity increased rapidly during 6–15 days after inoculation. However, it was hardly detected in the control medium throughout the cultivation. The production of the other carbohydrases such as amylase and CMCase in this mushroom was also stimulated when cultured with CNF-HWSF components (data not shown). Furthermore, these effects by CNF-HWSF were shown on other mushroom fungi (*H. marmoreus*, *P. ostreatus* and *F. velutipes*).

Gel filtrate analysis of the 500-L fraction from CNF-HWSF and the effect of the fraction on mycelial growth and xylanase activity of *L. edodes*

In our previous work and this experiment, the promoting effects on mycelial growth and extracellular enzyme productions were shown from the low molecular weight (500-L) fraction prepared from CNF-HWSF (Arai et al. 2003b). To search for the promoting components of a 500-L fraction, the crude product was extracted with organic solvents (hexane, benzene, diethyl ether, dichloromethane, and chloroform). As a result, only the water layer, removing organic solvent-soluble components from the 500-L fraction, was included in the promoting component. Then, the water layer was fractionated by gel filtration on a Sephadex G-10 column; the effect of these fractions on the mycelial growth of *L. edodes* was shown by assay (Fig. 3). Fractions C and D obtained from gel filtration increased the mycelial growth of *L. edodes* and stimulated xylanase activity in the medium. Fraction C included reducing sugars and amino acids, while these were not detected in fraction D.

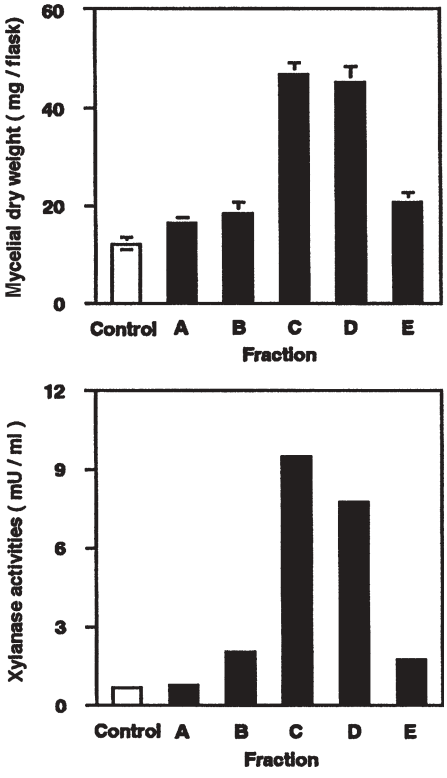

Fig. 3. Influence of the gel filtration fraction from CNF-HWSF components on vegetative mycelial growth and xylanase activity of *L. edodes*. Vegetative mycelia were cultured for 15 days at 24°C in a PDL medium added to each fraction (20%, 500-L and water layer; 40%, gel filtration samples). Values are means ± SD ($n = 5$). Xylanase activities were measured as described in Materials and methods. Enzyme activities were measured using a culture filtrate after 15 days of incubation. *Symbols* indicate fraction number by gel filtration using Sephadex G-10: *A*, 1–20; *B*, 21–24; *C*, 25–34; *D*, 35–60; *E*, 60–80

Discussion

Many enzymes are involved in the growth and development processes of fungi, and the ability of the mushroom to produce the decomposition enzymes necessary to degrade individual components of the growth substrate is important (Cai et al. 1999). Elucidation of cultivation and growth physiology mechanisms of mushrooms seems to be possible by examining the properties and behavior of these degradation enzymes.

We examined the effects of the CNF-HWSF ingredients on extracellular enzymes in *L. edodes*. CNF-HWSF was stimulated to produce degradation enzymes in the fungus,

and the effects were shown on other three fungal species. We previously reported that the promoting effects on mycelial growth were shown from the low molecular weight fractions (less than MW 500) prepared from CNF-HWSF (Arai et al. 2003b), and increase of carbohydrase activities on a medium was also shown in the low molecular weight fraction from CNF-HWSF in this experiment. These results suggest that a 500 or lesser fraction involves the main promoting components and that enzyme activation by CNF-HWSF concerns the promoting effect on mycelial growth with CNF-HWSF.

Secretion of xylanase and cellulases from fungi was induced with degradation substrate of these enzymes, and the production of substrates was regulated by the presence of glucose (Eriksson and Hamp 1978; Canevascini et al. 1979; Chow et al. 1994; Yagüe et al. 1997; Özcan et al. 1997). However, the production of enzymes from fungi was not stimulated with the polysaccharide substrates, and their degradation components have no catabolite repression with glucose (data not shown). De Groot et al (1998) reported that the expression of the xylanase and cellulase gene is regulated by components of the compost rather than by the substrates they act upon in *Agaricus bisporus*, which was the same result as ours.

Growth promotion of *L. edodes* appeared on both fraction C and D from gel filtrate analysis; the former included sugars and amino acids, and the latter has nothing. These results suggested that another promoting component was present other than reducing sugars and amino acids. Boyle (1998) described that the nitrogen source in the medium was responsible for the mycelial growth increases of *L. edodes* because carbon sources that did not contain nitrogen sources were ineffective. Although it is well known that *Armillaria* grows vigorously and produces abundant rhizomorphs when cultured on medium that is supplemented with low-concentration alcohol (Weinhold 1963), it was reported that no extension of mycelia was observed in the absence of amino acids, and carbon sources were utilized for growth only when amino acids existed in the medium with alcohol (Weinhold and Garraway 1966). The amino acids in CNF-HWSF were not the direct promoting component; it seems that the amino acids have the possibility to support activation of promoting components.

Mycelial weights in *L. edodes* were higher with CNF-HWSF and with a 500 or lesser fraction from the initial stage, but the biomass value on CNF-HWSF on the 15th day after inoculation remarkably rose beyond that on addition of the 500-L fraction. Mycelial dry weights of fungi were not increased when only the ethanol treatment precipitate (polymer) fraction from CNF-HWSF was added to a culture medium. However, the mixture of the high and low molecular fraction enhanced the mycelial growth (Arai et al. 2003b). Because CNF-HWSF contained rich nutrients (monomers and polymers) and the 500-L fraction included no or few polymers, growth enhancement also had a relation to the polymer components of CNF-HWSF as the nutritive components.

From the results presented here, the promoting mechanism on mycelial growth of mushrooms can be discussed as follows. Mycelial growth was enhanced by free amino acids (alanine, leucine, arginine, and others) and monosaccharides (glucose, arabinose, and xylose) in CNF-HWSF, reported previously as fast development, and a second promotion was caused from increase of supply with utilizable growth substrate by activation and stimulation of growth substrate degradation enzymes by some ingredients of CNF-HWSF.

In this report, we indicated that the D fraction by gel filtration has enhanced components other than sugar and amino acids, and the fraction stimulated to produce degradation enzymes in the mushroom. There is, in fact, a relationship between growth enhancements to increase enzymes. The main promotive component for growth of mushrooms exists in the D fraction from gel filtration. Further attempts will be made to investigate the isolation and characterization of promoting substances in CNF-HWSF to clarify the promoting mechanisms on edible mushrooms.

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