Occurrence of a Specific Protein in Basidiomycete-lytic Enzyme Preparation Produced by Bacillus circulans KA-304 Inductively with a Cell-wall Preparation of Schizophyllum commune

Shigekazu Yano, Sachiko Yamamoto, Toshikko Toge, Mamoru Wakayama, and Takashi TachiKi†

Department of Bioscience and Biotechnology, Faculty of Science and Technology, Ritsumeikan University, Shiga 525-8577, Japan

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KA-prep, a culture filtrate of Bacillus circulans KA-304 grown on a cell-wall preparation (CWP) of Schizophyllum commune, has been reported to have an activity to form protoplasts from S. commune mycelia. The SDS-polyacrylamide gel electrophoreses described here demonstrated that a specific proteinous component (molecular weight: 150,000) occurred in KA-prep. The protein (P150T) was also formed in culture filtrates with CWP of several basidiomycetes, which could release the protoplasts, suggesting that the component was an indispensable factor for protoplast formation. P150T, isolated from an ammonium sulfate fraction of KA-prep (0-30% saturation), did not have any protoplast-forming activity. Results were obtained indicating that P150T participates in protoplast formation together with chitinase(s) and β-glucanase(s) in KA-prep. The N-terminal amino acid sequence indicated an analogy of P150T to mutanase (α-1,3-glucanase) from Bacillus sp. RM1, and actually P150T hydrolyzed mutan as well as S-(α-1,3)-glucan from S. commune.

Key words: Bacillus circulans KA-304; Schizophyllum commune; protoplast formation; α-1,3-glucanase

Basidiomycetes are valuable not only as food or feed but also as a microbial resource for production of various bioactive substances, for transformation of biomass, and for improvement of polluted environments. Therefore, besides searching for new suitable species, breeding of known species with modern biotechnology has become significant, which requires efficient formation of reproducible protoplasts. However, conventional procedures with combination of commercial enzymes were usually complicated with low yields of reproducible protoplasts, indicating the need of further development and information on lytic enzymes.

In our previous paper, we found that a culture filtrate of Bacillus circulans KA-304 had an activity to form protoplasts from intact myelia of Schizophyllum commune when the bacterium was grown on a cell-wall preparation (CWP) of S. commune as an inducer. S. commune cell-wall has been reported to consist of a water-soluble β-1,3,1,6-glucan in the outer layer, an alkaline-soluble α-1,3,1,6-glucan (S-glucan) in the middle layer, and an alkaline-insoluble highly branched β-1,3,1,6-glucan (R-glucan) and chitin in the inner layer. As expected from the polysaccharide constituents in the cell wall, the culture filtrate with the CWP (KA-prep) contained several α-glucan-hydrolyzing enzymes (α-glucanase), β-glucan-hydrolyzing enzymes (β-glucanase), and colloidal chitin-hydrolyzing enzymes (chitinase). Most of these enzymes occurred in an ammonium sulfate fraction of 30-65 (70%) saturation of KA-prep whereas the protoplast-forming activity became almost negligible in the ammonium sulfate fractions (for example, 0-30%, 30-60% and 60-90% saturation). Some of the glycosidases have been isolated from the ammonium sulfate fraction of 30-65% saturation, and characterized to elucidate hydrolysis of the cell-wall. The addition of the isolated enzymes increased the protoplast-forming activity of KA-prep, suggesting their participation in the protoplast formation. However, the results were not enough to understand the lyses of the cell-wall exactly because the isolated enzymes did not show any protoplast-forming activity in the single or mixed state, which indicated the need of further analysis of components in KA-prep.

As described above, the protoplast-forming activity of KA-prep became almost negligible upon ammonium sulfate fractionation. However, later precise experiments using larger amounts of the ammonium sulfate fractions revealed a certain protoplast-forming activity in the fraction of 0-30% saturation, and a specific proteinous component (molecular weight

† To whom correspondence should be addressed. Tel: +81-77-566-1111 ex. 8265; Fax: +81-77-561-2659; E-mail: tachiki@se.ritsumei.ac.jp

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150,000, tentatively named P150T) was detected in KA-prep and in the ammonium sulfate fraction of 0–30% saturation. In addition, P150T was found in other culture filtrates, which were active in protoplast formation. These findings indicated that P150T might be a new factor, which was concerned closely with the protoplast formation.

This paper deals with demonstration, formation, isolation, and identification of P150T, and discusses its role in protoplast formation.

Materials and Methods

Microorganisms and cultivation. B. circulans KA-304 was grown at 30° C on Medium B consisting of 0.5% polypepton, 0.5% yeast extract, 0.1% K2HPO4, 0.03% MgSO4·7H2O, 0.5% NaCl, and 0.5% carbon source (as an inducer), pH 7. The centrifugal supernatant of the culture was used as a crude enzyme preparation.

To obtain mycelia for assaying protoplast-forming activity, Schizophyllum commune IFO 4928 was grown at 30° C with shaking on Medium C containing 2% glucose, 1% polypepton, 0.3% yeast extract, 0.3% K2HPO4, and 5 μg/l thiamine, pH 7. The mycelia pellet after 4 days of cultivation with 5 ml of Medium C in a test tube was fragmented with a blender (Waring No. 7009) at full speed for 1 min. The suspension was inoculated into 250 ml of Medium C in a 500-ml Sakaguchi flask, and incubated at 30° C on a reciprocal shaker for 1–2 days (100 strokes/min). The fine mycelial pellets were collected by filtration with cotton cloth, and washed several times with water, twice with 50 mM potassium phosphate buffer (pH 6.5), and finally with the same buffer containing 0.5 mM mannitol as an osmotic stabilizer.

CWP of S. commune and other basidiomycetes. CWP of S. commune was prepared as described previously.4 In the case of other basidiomycetes (Agaricus bisporus white- and brown-type, Flammulina velutipes, Hypsizigus marmoreus, and Pleurotus eryngii), their commercial fruiting bodies were used for preparing CWP by the same procedure as that for CWP of S. commune.

Assay of glycosidases. Two kinds of enzyme activity were assayed. (1) p-Nitrophenyl (pNP)-glycoside-hydrolyzing enzyme: The activity toward pNP-β-D-glucoside, pNP-α-D-glucoside, or pNP-β-D-N-acetylglucosaminide was assayed at 30° C by the method described previously.4 One unit of each enzyme was defined as the enzyme amount releasing 1 nmol of p-nitrophenol per min. (2) Polysaccharide-hydrolyzing enzyme (glycanase): A reaction mixture (0.4 ml) containing laminarin (2%), dextran (2%), or colloidal chitin (final turbidity at 610 nm, 0.4), 50 mM potassium phosphate buffer (pH 6.5), and appropriate amounts of enzyme was incubated at 30°C. The reaction was stopped by immersing the mixture in boiling water for 3 min, and reducing sugar formed was measured as glucose by the method of Somogyi and Nelson (in the case of laminarin or dextran as a substrate),13 or as N-acetylgalcosamine by the method of Shales10 or a modification of the method of Reissig (colloidal chitin as a substrate). Each enzyme was described as β-glucanase, α-glucanase, or chitinase in this paper, and one unit of the enzyme was defined as the amount that released 1 nmol of reducing sugar per min. Laminarin was used as a substrate for β-glucanase because of its structural similarity to the R-glucan of S. commune. Dextran (main α-1,6 linkage with side linkage of α-1,3) was used for examining α-glucanase activity despite its structural difference from the S-glucan of S. commune (main α-1,3 linkage with side linkage of α-1,6): α-glucan of similar structure as that of S-glucan could not be purchased commercially.

Assay of protoplast-forming activity. The activity was measured by the methods described by Mizuno et al.:4 the washed S. commune mycelia (0.2 g of fresh weight) were suspended in a mixture (1 ml) containing 50 mM potassium phosphate buffer (pH 6.5), 0.2 mg/ml sodium azide, 0.5 mM mannitol, and the enzyme preparation. After incubation at 30°C with moderate shaking (100 rpm), the released protoplasts were counted microscopically with a haemocytometer.

Analytical method. Protein was measured by Lowry’s method14 with egg albumin as a standard. In column chromatography, it was followed by the absorbance at 280 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli.15

Purification of P150T. All operations were done at 4–15°C, and 10 mM potassium phosphate buffer (pH 7.0) was used unless otherwise stated. P150T was detected by SDS-PAGE.

Step 1. B. circulans KA-304 was cultivated in 2-liter Sakaguchi flasks on a reciprocal shaker for 6 days with 1 liter of medium B containing 0.5% CWP of S. commune. The culture (5 liters) was centrifuged at 12,000 x g for 20 min.

Step 2. To the culture filtrate, was added solid ammonium sulfate to reach 90% saturation. After the mixture was stored overnight, the precipitate collected by centrifugation (12,000 x g for 20 min) was dissolved in and dialyzed against the buffer.

Step 3. The dialyzed solution was fractionated with ammonium sulfate, and the fraction of 0–30% saturation was dialyzed against the buffer.

Step 4. The dialyzed solution was put on a
DEAE-cellulose A-500 column (3 × 25 cm) equilibrated with the buffer. P150T was found in the unadsorbed fractions. The fractions were combined, and concentrated with ammonium sulfate (50% saturation).

Step 5. The precipitate was dissolved in and dialyzed against the buffer. The dialyzed solution was put on a hydroxyapatite column (3 × 25 cm) equilibrated with the same buffer. The unadsorbed fractions containing P150T were concentrated with ammonium sulfate (50% saturation).

Step 6. The precipitate was dissolved in a small volume of the buffer, and then filtered through a Sephrose CL-6B column (3 × 140 cm, 4 ml/tube). P150T was eluted in the fractions of No. 215-230, which were combined and concentrated with ammonium sulfate (50% saturation).

N-Terminal amino acid sequence. After SDS-PAGE, the purified protein band was transferred onto poly-vinylidene difluoride membrane by electroblotting. The blotted protein was cut out from the membrane, and the N-terminal amino acid sequence was analyzed according to the method of Edman using the peptide sequencer PPSQ-10 (Shimadzu).

Reagents. S-glucan was prepared from S. commune by the method described previously, and colloidal chitin by the method of Berger and Renolds. Mutan was a kind gift of Professor Shin-ichi Kinoshita, Hokkaido University. pNP-Glycosides were purchased from Nacalai Tesque and Sigma Chemical Co. Pachyman (Porio cocos) and pustulan (Unblicitaria papulass) were supplied by Calbiochem-Novabiochem Co., and laminarin (Laminaria digitata), chitin (crab shell), chitosan (crab shell), and cellulose were by Nacalai Tesque. Curdlan (Alcaligenes faecalis), starch (potato), and dextran were purchased from Wako Pure Chemical Co. Other reagents were the chemically pure grade of commercial products.

Result and Discussion

Occurrence of a specific component in culture filtrates active in protoplast-formation

When B. circulans KA-304 was grown on CWP of S. commune as an inducer, the culture filtrate (KA-prep) had an activity to form protoplasts from S. commune mycelia. The protoplast-forming activity was not found in a culture filtrate without CWP (KA-none), which showed low glycosidase activities.

Comparison of KA-prep and KA-none by SDS-PAGE (Fig. 1, lanes 1 and 2) indicated that a specific component occurred in KA-prep (molecular weight, about 150,000). The component (P150T) was not in other culture filtrates inactive in protoplast-formation (Fig. 1, lanes 3 to 7), which were obtained with polysaccharides of similar linkage type to those of the cell-wall polysaccharides of S. commune: usually hydrolyzing activity toward the polysaccharide increased in the filtrate.

P150T was also found in the culture filtrates with CWP of several basidiomycetes, which could release protoplasts from S. commune mycelia (Fig. 2). It was noticeable that the density of the protein band of the component in the SDS-PAGE gel (Fig. 2A) seemed to correlate with the protoplast-forming activity of the filtrates (Fig. 2B): the activities in the filtrates with Agaricus bisporus-CWP (lane 2) and with Hypsizigus marmoreus-CWP (lane 3) were almost the same as that of KA-prep (lane 1), and the activities of the others were less.

Occurrence of the P150T only in the culture filtrates active in protoplast formation suggested that the protein might be an indispensable factor for protoplast formation.

Analysis of KA-prep by ammonium sulfate fractionation

As described in the introductionary paragraph, most of the glycosidases in KA-prep were recovered in its ammonium sulfate fraction of 30–65 (70%) saturation. The protoplast-forming activity decreased to be almost negligible in the ammonium sulfate fractions of 0–30%, 30–60%, and 60–90% saturation. However, a later experiment using larger amounts of the fractions made it possible to detect a certain protoplast-forming activity in the fraction of 0–30% saturation (Table 1), which was about one third of the activity of the fraction of 0–90% saturation. From another point of view, Table 1 indicates that
the protoplast-forming activity of the ammonium sulfate fraction of 0-30% saturation increased by the addition of the fraction of 30-90% saturation, which contains most of the glycosidases in KA-prep. In addition, a SDS-PAGE demonstrated that the fraction of 0-30% saturation contained P150T (see, lane 4 of Fig. 4).

Figure 3A illustrates the distribution of glycosidases and P150T in the ammonium sulfate fractions. Figure 3B compares the protoplast-forming activity of the ammonium sulfate fractions of KA-prep, indicating that the activity rose from the fraction of 0-30% saturation to that of 0-90% saturation.

These figures suggest that P150T participates in the protoplast formation at least together with chitinase(s) and β-glucanase(s): the protoplast-forming activity of the fraction of 0-30% saturation might be displayed by a combination of P150T with small amounts of chitinase(s) and β-glucanase(s) in the fraction (see Table 1), and the activity was increased about 2-fold in the fraction of 0-50% saturation, for example, by the increase of chitinase(s), β-glucanase(s), and pNP-β-D-glucoside-hydrolyzing enzyme(s), which were in the fraction of 30-50% saturation.

Isolation and characterization of P150T
P150T (101 mg protein) was obtained from 5 liters of KA-prep (15,300 mg protein). SDS-PAGE (Fig. 4) showed the presence of a single protein in the final
preparation of Sepharose CL-6B column chromatography.

During the purification, the protoplast-forming activity of the ammonium sulfate fraction of 0–30% saturation decreased step by step, and the final P150T preparation had no activity. However, as shown in Table 2, an observation was made that a protoplast-forming activity appeared when P150T was added to KA-none, which has been inactive in protoplast formation. P150T also gave the protoplast-forming activity to the ammonium sulfate fraction of 30–50% saturation, which is inactive in protoplast-formation whereas it contained certain amounts of chitinase(s), and β-glucanase(s) (see Fig. 3 and Table 1). The amounts of β-glucanase(s), chitinase(s), and pNP-β-D-glucoside-hydrolyzing enzyme(s) in each reaction mixture (see, the legend for Table 2) were seemingly incompatible with the number of protoplasts formed in the mixture. However, such finding might be not so unusual in a complex reaction like protoplast formation, where several enzymes (variable in species and amounts) react toward complex substrates: such a reaction could not proceed as simply as a reaction with single enzyme(s) and simple substrate(s).

The ability of P150T to give the protoplast-forming activity to KA-none or the ammonium sulfate fraction of 30–50% saturation was lost by 5-min heating in boiling water, indicating its proteinous property.

The N-terminal sequence of P150T was A G G P N L T L G K T I T A S G Q S (18 residues) with an 88% sequence identity to those of a mutanase from Bacillus sp. RM1 (A G G P N L T P G K P I T A S G Q S). The differences were the 8th and the 11th amino acids. Mutanase is an enzyme hydrolyzing mutan (α-1,3-glucan with some α-1,6-glucan side chains), a major component of exo-polysaccharides produced by tooth-colonizing streptococci such as Streptococcus mutans.

As suggested by its analogy to mutanase in N-terminal sequence, P150T actually hydrolyzed mutan (Fig. 5), and also showed certain hydrolyzing activity on S-glucan prepared from S. commum cell-wall. However, release of reducing sugar from CWPrep of S. commum was not so obvious (data, not shown). P150T was inactive to pNP-α-D-glucoside, dextran or starch (data not shown).

The substrate specificity indicated that P150T was an endo-type α-glucanase with affinity for α-1,3-linkages. Occurrence of mutanase has been reported in

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**Table 2. Effects of P150T on the Protoplast Formation with KA-none or Ammonium Sulfate Fraction of KA-prep (30–50% saturation)**

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>Protoplast formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA-prep b)</td>
<td>100</td>
</tr>
<tr>
<td>KA-none b)</td>
<td>0</td>
</tr>
<tr>
<td>P150T 0.2 mg</td>
<td>0</td>
</tr>
<tr>
<td>KA-none + P150T 0.2 mg</td>
<td>67</td>
</tr>
<tr>
<td>KA-none + P150T 0.1 mg</td>
<td>53</td>
</tr>
<tr>
<td>KA-none + P150T 0.05 mg</td>
<td>15</td>
</tr>
<tr>
<td>Ammonium sulfate fraction 30–50% b) + P150T 0.2 mg</td>
<td>78</td>
</tr>
</tbody>
</table>

a) KA-prep and KA-none were concentrated 10-times with ammonium sulfate (90% saturation).
b) KA-prep was fractionated with ammonium sulfate (30–50% saturation), and the fraction was concentrated 10 times by the same methods as described in Table 1.
c) Protoplast formation was done for 6 h with 0.5 ml of the concentrated enzyme preparation with or without several concentrations of P150T in 1 ml of the reaction mixture. Released protoplasts were counted, and the number was indicated relatively to that formed by KA-prep.

The amounts of β-glucanase, chitinase and pNP-β-D-glucoside-hydrolyzing enzyme were: 58, 75 and 78 units in the mixture with KA-prep; 16, 28 and 8 units in the mixture with KA-none; 26, 32 and 38 units in the mixture with the ammonium sulfate fraction of 30–50% saturation.

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**Fig. 4.** SDS-PAGE of purified P150T.

The preparation of each purification step was analyzed by SDS-PAGE. Lane 1, molecular marker; lane 2, KA-none; lane 3, KA-prep (starting material); lane 4, ammonium sulfate fraction of 0–30% saturation; lane 5, DEAE-cellulose chromatography; lane 6, hydroxyapatite chromatography; lane 7, Sepharose CL-6B chromatography (final preparation). Arrow indicates P150T.

**Fig. 5.** Hydrolysis of Mutan and S-Glucan by P150T.

The hydrolyzing activity was measured in a reaction mixture consisting of 1% mutan (○) or S-glucan of S. commum (●), 50 mM potassium phosphate buffer (pH 6.5) and P150T (0.25 mg/ml). Incubation was done at 30°C.
Trichoderma viride, Trichoderma harzianum, Aspergillus nidulans Flavobacterium sp., Streptomyces sp., Pseudomonas sp., Cladosporium resinae, Streptomyces chartreusse, Bacteroides oralis, and Bacillus circulans HU-M1. Among them, the enzyme of B. circulans HU-M1 was similar to P150T in respect to the molecular weight (160,000 to 150,000 of P150T).

In summary, this paper reported that P150T in KA-prep, an indispensable factor for protoplast formation from S. commune mycelia, was an α-1,3-glucan-hydrolyzing enzyme. P150T was formed by B. circulans KA-304 inductively with CWP of S. commune or several basidiomycetes. P150T gave the protoplast-forming activity to the ammonium sulfate fraction of 30-50% saturation, which was inactive in the protoplast formation whereas it contained quite a part of chitinase(s) and β-glucanase(s) in KA-prep. These results implied that P150T (α-1,3-glucan-hydrolyzing enzyme) and the two glycosidases did the protoplast formation.

Purification and characterization of chitinase(s) and β-glucanase(s) in the ammonium sulfate fraction of 30-50% saturation are now undertaken in respect to protoplast formation.

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