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A novel thermostable α-1,3-glucanase from *Streptomyces thermodiastaticus* HF 3-3

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Wasana Suyotha,[1] Hidehisa Fujiki,[2] Vipavee Cherdvorapong,[2] Kazuyoshi Takagi,[3] Shigekazu Yano,[4] and Mamoru Wakayama[2,*]

[1] *Biotechnology for Bioresource Utilization Laboratory, Department of Industrial Biotechnology, Faculty of Agro-industry, Prince of Songkla University, Hat Yai 90112, Thailand*
[2] *Department of Biotechnology, Faculty of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan*
[3] *Department of Applied Chemistry, Faculty of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan*
[4] *Department of Biochemical Engineering, Graduate School of Sciences and Engineering, Yamagata University, Jonan, Yonezawa, Yamagata 992-8510, Japan*

Thermally stable α-1,3-glucanase HF65 was purified from culture filtrate of *Streptomyces thermodiastaticus* HF3-3. The molecular mass of this enzyme was estimated to be 65 kDa and 45.7 kDa by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography, respectively. The purified enzyme retained more than 50% of maximum activity even after incubation at 65°C more than 2 h. Moreover, α-1,3-glucanase HF65 was stable in the presence of chemicals like SDS, benzethonium chloride, and sodium fluoride at a concentration of 1%. The enzyme also exhibited salt tolerance at a concentration up to 20%. The observed stability of α-1,3-glucanase HF65 to salt and surfactants is a great advantage for its addition to commercial oral care products. Interestingly, the *N*-terminal amino acid sequence did not show any similarity to those of known α-1,3-glucanases, while the sequence of internal eight amino acid residues of this enzyme was homologous with those of mycodextranases. Nevertheless, the enzyme exhibited high specificity against α-1,3-glucan. According to these results, the enzyme purified from *S. thermodiastaticus* HF3-3 was classified as α-1,3-glucanase which was highly homologous to mycodextranase in amino acid sequence.

Key Words: α-1,3-glucanase; mycodextranase; *Streptomyces thermodiastaticus*; thermal stability

Introduction

α-1,3-Glucanases is an enzyme that catalyzes the hydrolysis of α-1,3-glycosidic bond of α-1,3-glucan, which is the main component of extracellular polysaccharides produced by oral *Streptococci* in dental plaque (Li and Bowden, 1994; Yakushiji et al., 1984) and the cell wall of some fungi (Hochstenbach et al., 1998; Sietsma and Wessel, 1977; Zonneveld, 1972). Recently, α-1,3-glucan was also recognized as a virulence factor of some pathogenic fungi (Fujikawa et al., 2009, 2012; Rappleye et al., 2007). Thus, the enzyme is of interest due to its potential use in the removal of dental plaque and the biological control of pathogenic fungi. Some studies have demonstrated the ability of α-1,3-glucanases to reduce the plaque synthesized by *Streptococcus* species, as well as to inhibit plaque formation (Fuglsang et al., 2000; Pleszczyńska et al., 2010; Shimotsuura et al., 2008; Tsumori et al., 2011; Wiater et al., 2004, 2008). Moreover, they indicated that the degradation of plaque by the enzyme was more effectively performed in combination with dextranase (Fuglsang et al., 2000; Shimotsuura et al., 2008). From the viewpoint of controlling pathogenic fungi, Fujikawa et al. (2009, 2012) showed that α-1,3-glucanase was required to prevent the infection of plant pathogens, such as *Magnaporthe oryzae*, *Cochioborus miyabeanus*, and *Rhizoctonia solani*, that accumulated α-1,3-glucan on their cell surface to avoid the defense system of the plants. They also demonstrated that the lysis of germ tubes was achieved by the combined action of chitinase and α-1,3-glucanase. In previous studies, we also reported a similar effect of α-

*Corresponding author: Mamoru Wakayama, Department of Biotechnology, Faculty of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan.
Tel: +81-77-566-1111 Fax: +81-77-561-2659 E-mail: wakayama@sk.ritsumei.ac.jp

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1,3-glucanase on degrading the cell wall of *Schizophyllum commune* (Suyotha et al., 2013, 2014; Yano et al., 2003).

α-1,3-Glucanase is distributed among bacteria, yeast and fungi. The enzymes isolated from bacteria and fungi were purified and their properties were investigated for their future use as an anticariogenic agent and a biological control agent of pathogenic fungi. However, the commercial use of this enzyme is still limited for several reasons. For example, there is a problem of enzyme production, because it can only be inducible by α-1,3-glucan substrates, which are not an abundant natural polymer, such as cellulose and chitin (Imai et al., 1977; Meyer and Phaff, 1980; Wiater et al., 2008; Yano et al., 2003). Another problem is the stability of the enzyme under the specific conditions conducted for oral care products, or a pesticide. Unfortunately, none of the characterized α-1,3-glucanases exhibit extreme stability, such as a high thermal stability, which is one of the properties desired for industrial utilization; almost all characterized α-1,3-glucanases were unstable above 65°C (Fuglsang et al., 2000; Imai et al., 1977; Meyer and Phaff, 1980; Pleszczyńska et al., 2010; Shimotsuura et al., 2008; Suyotha et al., 2013, 2014; Tsumori et al., 2011). In recent years, genetic manipulations have been used to develop the properties of target enzymes. However, because there is little biochemical and structural information for the α-1,3-glucanase group, enzyme improvement using reasonable protein engineering techniques is not available at present. Therefore, the screening of naturally-occurring microorganisms could still be the best way to obtain new strains which produce α-1,3-glucanase with the desired properties for commercial applications.

In the present study, a thermostable α-1,3-glucanase-producing bacterial strain was isolated and identified as *Streptomyces thermodiastaticus* HF3-3. The purified enzyme was more stable at 65°C and tolerant against a salt concentration of 0.25–1.0% (w/v) of salt and surfactants. Enzyme identification based on the similarity of internal amino acid sequences (eight amino acids) showed that it is homologous to mycrodextranase and has a high specificity towards α-1,3-glucan. This is the first report of a thermostable α-1,3-glucanase with a high sequence similarity to mycodextranase.

Materials and Methods

Isolation and screening of thermally stable α-1,3-glucanase-producing bacteria. The soil samples used in this study were collected from hot springs and forests in Japan. A soil sample was suspended in sterilized water and spread on an agar plate containing 0.5% α-1,3-glucan, 0.05% K_2HPO_4, 0.05% KH_2PO_4, 0.01% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, and 1.5% agar. After incubation at 50°C for 7 d, colonies that had formed clear zones on the plate were isolated. To screen thermostable α-1,3-glucanase-producing microorganisms, each isolate was inoculated into 5 mL of the medium, described above, in a test tube and incubated at 50°C for 3 d. The culture supernatant was collected by centrifigation at 10,000 × *g* at 4°C for 5 min and subsequently treated by heating at 60–70°C for 10 min before being used to measure α-1,3-glucanase activity.

16S ribosomal DNA analysis. 16S ribosomal DNA was amplified by polymerase chain reaction (PCR) using primers 20f (5′-AGTTTGATCCTGGCTC-3′) and 1510r (5′-GGCTACCTTGTTACGA-3′). PCR was performed in a reaction mixture containing 10 ng of chromosomal DNA, 20 pmol of each primer, 200 μM each of deoxynucleoside triphosphates (dNTP), and 2.5 units of Blend *Taq* polymerase (Takara, Shiga, Japan). Thermal cycling was 1 cycle of 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. The sequence of the PCR products was analyzed with an ABI-Prism Big Dye terminator cycle sequencing ready reaction kit and an ABI-prism 377 sequencer (Applied Biosystems, Tokyo, Japan). A homology search of the DNA sequences was carried out with the BLAST program of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

Production and purification of α-1,3-glucanases. The isolated strain HF3-3 was grown in a 100-mL baffle shake flask on a rotary shaker at 100 rpm and 50°C for 3 d with 20 mL of production medium containing 1.0% α-1,3-glucan, 0.05% K_2HPO_4, 0.05% KH_2PO_4, 0.01% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$ and 0.05% KCl. The culture was centrifuged at 10,000 × *g* for 10 min at 4°C. Solid ammonium sulfate was added to the supernatant to 80% saturation. After the mixture was stored at 4°C overnight, the precipitate collected by centrifugation (10,000 × *g* for 10 min at 4°C) was dissolved in, and dialyzed against, 10 mM Tris-HCl (pH 8.0) at 4°C. The dialysate was applied to a DEAE-cellulofine column (7.5 × 1.8 cm) equilibrated with 10 mM Tris-HCl (pH 8.0). After washing with the same buffer, the column was developed with a series of stepwise elution using a buffer containing 75, 100, 125, and 150 mM NaCl. The enzymes were eluted with the buffer containing 100 mM NaCl. The active fractions were collected and dialyzed against 10 mM potassium phosphate buffer (pH 7.0). Ammonium sulfate was added to the dialysate to 25% saturation, and the solution was applied to a Butyl-Toyopearl 650M column (7.5 × 1.3 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 25% ammonium sulfate. After washing with the same buffer, the column was developed with the buffer containing 20% ammonium sulfate for elution of the enzyme. The active fractions were collected and dialyzed against 10 mM citrate buffer (pH 5.5).

Determination of molecular mass. The molecular weight of the purified enzyme was estimated in both denatured and native conditions. The molecular weight of the denatured protein was determined on 10% SDS-PAGE as described by Laemmli (1970). Pre-stained Protein Markers Broad Range (Nacalai Tesque, Japan) were used as standards. The molecular mass of the native protein was also determined by size exclusion chromatography using a Superdex HR 10/30 column (1 × 30 cm). The colunm was equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. The proteins were eluted with the same buffer at a flow rate of 0.4 mL/min. The standard proteins used were glutamate dehydrogenase from yeast (MW 290,000), lactate dehydrogenase from yeast (MW 140,000), enolase from yeast (MW 67,000), myoki-

nase from yeast (MW 32,000), and cyotochrome *c* from equine heart (MW 12,400).

Assay for α-1,3-glucanase activity. The activity was determined according to the methods described previously (Suyotha et al., 2013). The reaction mixture containing 1% α-1,3-glucan, 50 mM citrate buffer (pH 5.5), and the appropriate amount of enzyme was incubated at 50°C for 1 h. One unit of the enzyme was defined as the amount of enzyme releasing 1 μmol of reducing sugar (as glucose) per min.

Assay for protein. The protein concentration of each fraction in the purification steps was measured by the absorbance at 280 nm. The total protein concentration of the enzyme was estimated by Lowry's method with egg albumin as standard (Lowry et al., 1951).

Thin-layer chromatography. Hydrolysis products by α-1,3-glucanase was analyzed by thin-layer chromatography (TLC). The hydrolysis products obtained in the reaction mixture for 10 min to 24 h at 30°C were spotted on a TLC Silica gel 60 (Merck, Darmstadt, Germany) and developed with a solvent of n-butanol: acetic acid: distilled water (2:1:1 v/v). The sugar spots on the plate were stained by spraying with 0.2% (v/v) orcinol in sulfuric acid and heated at 100°C for 10 min.

Analysis of N-terminal and internal amino acid sequences. To identify the purified protein, both *N*-terminal and internal amino acid sequences were analyzed. To determine the internal amino acid sequences, the purified protein was digested by endopeptidase. The mixture containing 20 μl of the purified enzyme and 5 μl of 10% SDS was heated with boiling water for 5 min. After cooling, 5 μl of ammonium carbonate buffer (25 mM, pH 7.8) and 1 μl of lysyl-endopeptidase were added into the mixture and incubated at 37°C for 1 h. The protein samples with and without digestion by endopeptidase were individually separated on 10% SDS-PAGE and then transferred to a polyvinyldene difluride (PVDF) membrane. The protein bands on the membrane were excised and sent for analysis at Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

The effect of pH on the activity and stability of α-1,3-glucanase. The optimal pH for α-1,3-glucanase activity was determined by incubating the reaction mixture containing the enzyme, 1% α-1,3-glucan and 50 mM buffer of various pH values at 50°C for 1 h. To determine its pH stability, the enzyme was incubated at 65°C for 10 min in 50 mM buffers of various pHs. After treatment, α-1,3-glucanase activity was determined as using 1% α-1,3-glucan as the substrate. The buffers used were citrate buffer (pH 4.0–6.0), potassium phosphate buffer (pH 6.0–7.5), borated buffer (pH 7.5–9.5) and sodium carbonate buffer (pH 9.5–10.0).

The effect of temperature on the activity and stability of α-1,3-glucanase. To determine the optimal temperature, the activity was measured within the temperature range 30–75°C for 10 min in 50 mM citrate buffer (pH 5.5). The thermal stability was determined after treatment of the enzyme at various temperatures in the range 30–75°C for 10 min. The thermal tolerance of the enzyme was also investigated by incubating the enzyme at 60, 65 and 75°C for 2 h and measuring the residual activity at different given times.

The effect of salts and surfactants on the activity and stability of α-1,3-glucanase. The effect of the optimal salt concentration on the activity of α-1,3-glucanase was measured by incubating reaction mixtures with different concentrations of NaCl in the range 0–20% (w/v) at 50°C for 1 h. The salt tolerance was determined after incubation of the enzyme in NaCl solutions at different concentrations at 65°C for 10 min. After the treatment, the residual activity was measured. To determine the effects of salt and surfactants on α-1,3-glucanase activity, sodium fluoride (NaF), sodium dodecyl sulfate (SDS), and benzethonium chloride (BTC) at final concentrations of 0–1% were added into the reaction mixture containing 1% α-1,3-glucan, 50 mM citrate buffer (pH 5.5), and α-1,3-glucanase HF-3-3. After incubation at 50°C for 1 h, the enzyme activities were determined.

The effects of metal ions and chemical reagents on the activity of α-1,3-glucanase. The effect of metals on the activity of α-1,3-glucanase was investigated by adding monovalent ions such as Na^+, K^+, Li^+, and Ag^+ and divalent ions such as Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} in the reaction mixture at 1 mM concentrations. The influence of other chemicals was investigated using NH_4^+, EDTA, and DDT at 1 mM. After incubation at 50°C for 1 h, the enzyme activity was measured.

Reagents. α-1,3-Glucan was prepared with methods described previously (Suyotha et al., 2013). The other reagents were chemically pure grades of commercial products.

Results

Isolation and identification of thermostable α-1,3-glucanase-producing microorganisms

In primary screening, 76 strains grown on selective medium using α-1,3-glucan as a carbon source under incubation at 50°C were isolated from soil samples. To screen strains producing thermostable α-1,3-glucanase, the culture supernatant of each isolate was heated for 10 min at 60, 70 and 80°C, respectively, and then the residual α-1,3-glucanase activity was measured. Among 76 isolates, 3 strains produced thermostable α-1,3-glucanase that retained more than 90% of its full activity after incubation at 60°C. One strain (HF3-3) produced the highest thermostable α-1,3-glucanase, which exhibited about 95% of full activity after incubation at 65°C for 10 min. Even after treatment at 70°C for 120 min, the enzyme from this strain showed 20% of residual activity. Based on these results, the strain HF-3-3 was selected as a thermostable α-1,3-glucanase-producing organism for future study. The analysis of 16S ribosomal DNA sequence indicated that the strain HF-3-3 is most similar to *Streptomyces thermodiastaticus* strain JCM 4840 (99%) and *Streptomyces thermospinosisporus* (99%). On the basis of these findings, the strain FH11 was designated as *Streptomyces thermodiastaticus* HF3-3 in this study.

Table 1. Purification of α-1,3-glucanase HF65 from *Streptomyces thermodiastaticus* HF3-3.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	36.9	122	0.302	100	1
Ammonium sulfate concentration	6.4	20.7	0.308	17.3	1.02
DEAE-Cellufine	9.08	6.54	1.39	24.6	4.60
Butyl-TOYOPEARL	5.11	2.2	2.32	13.8	7.69

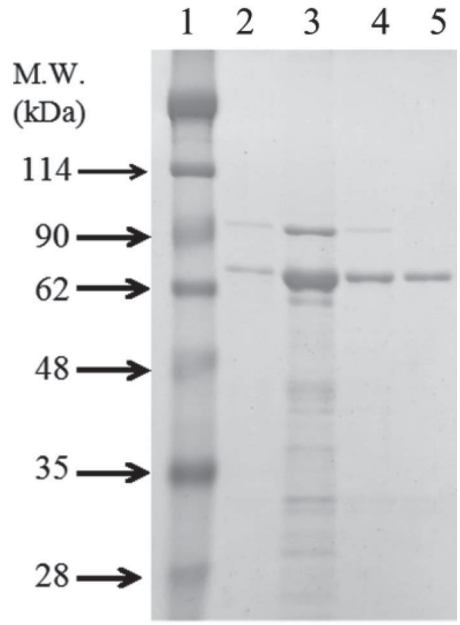

Fig. 1. SDS-PAGE.

10% SDS-PAGE was used and the gel was stained with Coomassie Brilliant Blue R-250. Lane1: Marker, Lane2: Culture filtrate, Lane3: Ammonium sulfate concentration, Lane4: DEAE-Cellufine, Lane5: Butyl-TOYOPEARL.

Production of α-1,3-glucanases from S. thermodiastaticus HF3-3

Several culture conditions were investigated for optimal production of α-1,3-glucanases by *S. thermodiastaticus* HF3-3. The cultivation of *S. thermodiastaticus* HF3-3 at 50°C for 3 d in a 100-mL Erlenmeyer flask with baffles containing 20 ml of basal medium (0.05% K_2HPO_4, 0.05% KH_2PO_4, 0.01% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$ with 1.0% α-1,3-glucan as inducer substrate was most suitable for α-1,3-glucanase production. In addition, enzyme production was also increased when *S. thermodiastaticus* HF3-3 was cultured in the medium supplemented with 0.0001% $FeSO_4 \cdot 7H_2O$ and 0.05% KCl. The optimal cultivation period for enzyme production was examined and the highest enzyme production was obtained on Day 3. Accordingly, the culture supernatant was collected after 3 d incubation for future purification.

Enzyme purification

α-1,3-glucanase was purified from the culture filtrate of *S. thermodiastaticus* HF3-3 using ammonium sulfate fractionation, DEAE-cellulofine A500 column chromatography, and Butyl-Toyopearl 650M column chromatography. As shown in Fig. 1, *S. thermodiastaticus* HF3-3 predominantly produced two proteins with the apparent molecular masses of 90 kDa and 65 kDa on SDS-PAGE, respectively. Two proteins were separated from each other by hydrophobic chromatography. One was eluted with the buffer containing 20% ammonium sulfate (designed as α-1,3-glucanase HF65) and the other one was eluted with the buffer containing 16% ammonium sulfate. Interestingly, both proteins exhibited α-1,3-glucanase activity (data not shown). In the present study, α-1,3-glucanase HF65 was characterized first and the purification results are summarized in Table 1. The enzyme was purified 7.69-fold with a specific activity of 2.32 U/mg and a recovery of 13.8%. The purified α-1,3-glucanase HF65 showed a single band on SDS-PAGE with an apparent molecular mass of 65 kDa. The molecular mass determined by size exclusion chromatography was 45.7 kDa. The lower molecular mass in gel filtration could be due to the interaction between the enzyme and chromatography resin that retarded the elution of the enzyme. This phenomenon is often found in polysaccharide-degrading enzymes (Silva et al., 1999; Takeda et al., 2000).

Determination of N-terminal and internal amino acid sequences

N-terminal and internal amino acid sequences of purified α-1,3-glucanase HF65 were determined to be AATAGADL and IGPDYTQG, respectively. These sequences were compared with other protein sequences available in NCBI protein database using the PSI-BLAST searching program (http://blast.ncbi.nlm.nih.gov/). The results revealed that *N*-terminal amino acid sequences of α-1,3-glucanase HF65 were homologous to that of glycosyltransferase from uncultured bacterium (Accession No.: ALS91666), 4-carboxymuconolactone decarboxylase from *Streptomyces* sp. PBH53 (Accession No.: AKN72939) and *N*-(5′-phosphoribose) anthranilate isomerase from *Chloroflexus* sp. Y-396-1 (Accession No.: WP_028457075), but it did not show any homology with the known α-1,3-glucanases. On the other hand, the internal amino acid sequnce of the enzyme was homologous to those of mycodextranases from *Streptomyces* sp. (Table 2). Therefore, it could be considered that α-1,3-glucanase HF65 from *S. thermodiastaticus* HF3-3 belongs to the family of mycodextranase.

Effects of pH and temperature on activity and stability of α-1,3-glucanase HF65

The effect of pH was determined using different pH buffers ranging from pH 4–10. The α-1,3-glucanase HF65 exhibited the maximum activity at pH 5.0–5.5 and greatly decreased activity in an alkali pH range (Fig. 2A). The

Table 2. Alignment of *N*-terminal and internal amino acid of the purified α-1,3-glucanase HF65 from *Streptomyces thermodiastaticus* HF3-3 with the other proteins.

Enzyme	*N*-terminal amino acid sequences	
α-1,3-Glucanase HF65 form *Streptomyces thermodiastaticus* HF3-3	: **AATAGADL**	:
N-(5′-phosphoribose) anthranilate isomerase from *Chloroflexus* sp. Y-396-1	: ALV**AATAGADL**I	: 23
Glycosyltransferase form uncultured bacterium	: DLI**AATAGADL**V	: 27
4-Carboxymuconolactone decarboxylase from *Streptomyces* sp. PBH53	: AIT**AATAGADL**T	: 19

Enzyme	Internal amino acid sequences	
α-1,3-Glucanase HF65 form *Streptomyces thermodiastaticus* HF3-3	: **IGPDYTQG**	
Mycodextranase from *Streptomyces* sp. NRRL B-24085 (WP_053853207)	: R**IGPDYTQG**TLASEASG	: 63
Mycodextranase from *Streptomyces collinus* Tu 365 (AGS72909)	: R**IGPDYTQG**TLASEASG	: 62
Mycodextranase from *Streptomyces phaeopurpureus* (KUM70241)	: R**IGPDYTQG**TLASEASG	: 68
Mycodextranase from *Streptomyces griseorubiginosus* (KUN67769)	: R**IGPDYTQG**TLASEASG	: 68
Mycodextranase from *Streptomyces* sp. AW19M42 (WP_024491542)	: K**IGPDYTQG**TLASEASG	: 80
Mycodextranase from *Streptomyces* sp. 150FB (WP_040025370)	: K**IGPDYTQG**SLASEASG	: 72
Mycodextranase from *Streptomyces* sp. CdTB01 (WP_058921973)	: K**IGPDYTQG**SLASEASG	: 68

Residues identical with that of α-1,3-glucanase HF65 form *S. thermodiastaticus* HF3-3 are indicated in bold black letters.

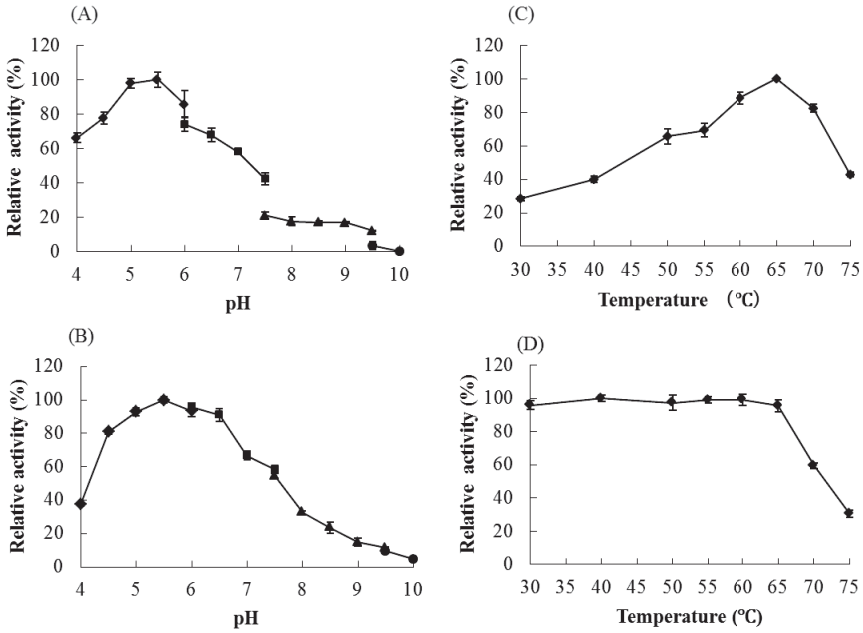

Fig. 2. Biochemical properties of α-1,3-glucanasses HF65 from *Streptomyces thermodiastaticus* HF3-3.

Optimal pH (A), pH stability (B), optimal temperature (C), and thermostability (D), were assayed as described in "Materials and Methods". The buffers used were citrate buffer (◆) for pH 4.0–6.0, potassium phosphate buffer (■) for pH 6.0–7.5, borate buffer (▲) for pH 7.5–9.5, and sodium carbonate buffer (●) for pH 9.5–10.0. Error bars represent mean values and standard deviation of triplicate determinations.

enzyme was stable over a pH range of 4.5–6.5 (Fig. 2B). The pH dependence of α-1,3-glucanase HF65 activity was almost similar to those of bacterial α-1,3-glucanases that have been reported to exhibit optimum activity at acidic pH regions (Fuglsang et al., 2000; Imai et al., 1977; Meyer and Phaff, 1980; Pleszczyńska et al., 2010; Shimotsuura et al., 2008; Suyotha et al., 2014; Tsumori et al., 2011). The effect of temperature was also investigated in the range 30–80°C (Fig. 2C). It was found that α-1,3-glucanase HF65 exhibited the highest activity at 65°C. The residual activity of the enzyme after incubation for 10 min at different temperatures was evaluated. The enzyme was stable up to 65°C and still remained 60% of full activity after the treatment at 70°C (Fig. 2D). Figure 3 shows that the enzyme retained more than 50% of its maximum activity even after incubation at 65°C for more than 2 h.

The effects of metal ions and chemical reagents on the activity of α-1,3-glucanase HF65

As shown in Fig. 4, ions such as Na^+, K^+, NH_4^+, Mg^{2+}, Fe^{2+}, Zn^{2+}, and Ni^{2+} did not significantly affect the α-1,3-glucanase HF65 activity. A significant increase in enzyme

activity was observed in the presence of 1 mM Co^{2+}, with relative activity of 160%. Li^{+}, Ca^{2+}, Mn^{2+}, and Ca^{2+} slightly stimulated the enzyme activity with relative activity of 115, 125, and 124%, respectively. These metal ions could be used as an activator to promote the catalytic efficiency of α-1,3-glucanase HF65 in applications. In contrast, the enzyme activity was strongly inhibited in the presence of Ag^{+}, losing more than 80% of its activity. A moderate inhibition was observed in the presence of Cu^{2+}, upon which the activity decreased to 80% of full activity. This is because heavy ions, such as Ag^{+} and Cu^{2+}, could inhibit the formation of a disulfide bond in protein by binding to an SH group of a side chain of cysteine (Zuazaga et al., 1984). Until now, no report has demonstrated the involvement of thiol groups in catalytic mechanism of α-1,3-glucanase or the other glycosidases. On the other hand, many studies have suggested the role of thiol groups in maintaining the architecture of the active site (Krajewska, 2008; Siddiqui et al., 2005). It is possible that α-1,3-glucanase HF65 might contain a disulfide bond that contributes to activity as well as thermal stability.

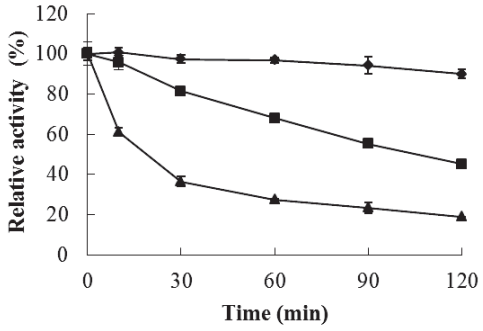

Fig. 3. Temperature stability of α-1,3-glucanase F65 at 60°C (◆), 65°C (■), and 70°C (▲).

The residual activity of the enzyme was measured at 50°C after the enzyme was treated at a given temperature for 10 min to 2 h in a reaction mixture comprised of 1% α-1,3-glucan and 50 mM citrate buffer (pH 5.5). Error bars represent mean values and standard deviation of triplicate determinations.

Effects of salts and surfactants on the activity and stability of α-1,3-glucanase HF65

For the future application of the enzyme in dental care, the effect of salts, and surfactants that are commonly added to toothpastes on enzyme activity, were examined. NaCl could be used as an astringent to prevent gingivitis and periodontitis (Wolinsky and Lott, 1986). In this study, the effect of NaCl on the activity and stability of α-1,3-glucanase HF 65 was examined in the concentration range 5–20%. Figure 5 shows that NaCl had no negative effect on the activity and stability of α-1,3-glucanase, and that the enzyme activity was slightly enhanced by increasing NaCl up to 5% (Fig. 5A), suggesting an optimum concentration in terms of ionic strength for enzyme activation with NaCl. Moreover, the enzyme retained full activity even after it was treated with a high ionic concentration (20% NaCl) under incubation at 65°C for 10 min (Fig. 5B).

In addition, the effect of surfactants, SDS, BTC, and NaF, on α-1,3-glucanase HF65 activity were examined over the concentration range 0.25–1%, which is usually set in commercial toothpastes. As shown in Fig. 6, no decrease in enzyme activity was observed in the presence of these reagents at given concentrations.

Substrate specificity and hydrolysis production

Glucan polysaccharides with different linkages such as α-1,3-glucan, dextran (α-1,6), cellulose (β-1,4), amylose (α-1,4), amylopectin (α-1,4 and α-1,6), and laminarin (β-1,3) were used to determine the substrate specificity of purified enzyme. Enzyme activity was only detected in the reaction containing α-1,3-glucan as a substrate, indicating that the purified enzyme has a high specificity towards α-1,3-glucan. Subsequently, hydrolysates produced from α-1,3-glucan were analyzed by TLC. After incubation for 1 h, disaccharide, trisaccharide, tetrasaccharide, and pentasaccharide were detected as main hydrolytic

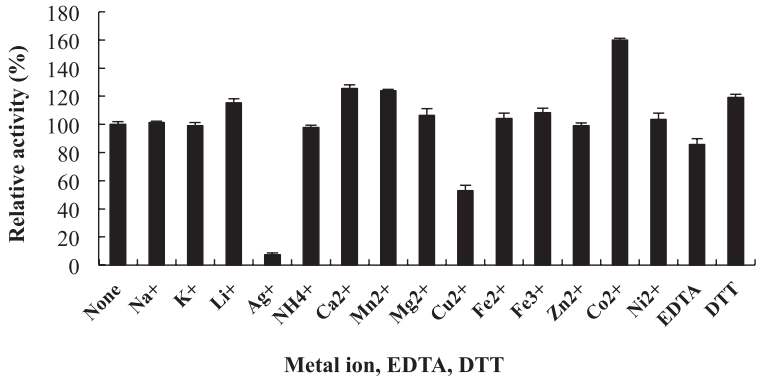

Fig. 4. Effect of metal ions and chemical reagents on α-1,3-glucanase HF65 activity.

The effects of metals and chemical reagents on the activity of α-1,3-glucanase HF65 were investigated by adding ions in the reaction mixture at 1 mM concentrations. After incubation at 50°C for 1 h, the enzyme activity was measured. Error bar represents mean values and standard deviation of triplicate determinations.

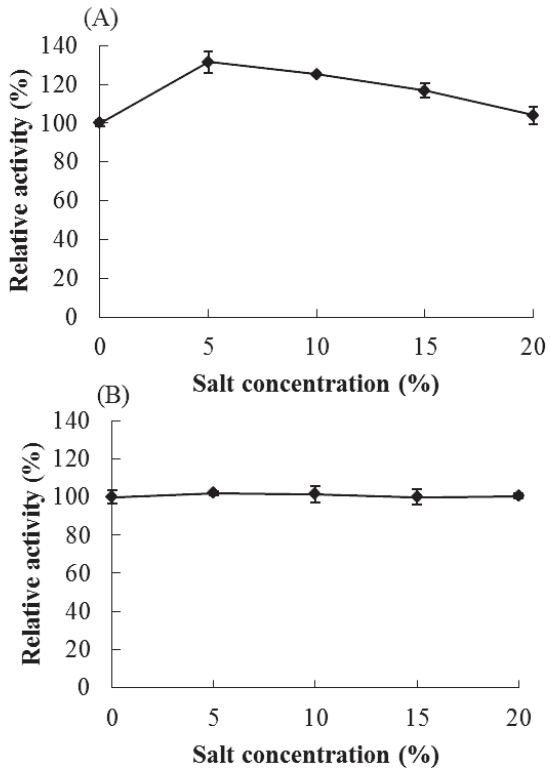

Fig. 5. Effect of NaCl (salt) concentration on activity (A) and stability (B) of α-1,3-glucanase HF65.

NaCl at different concentrations of 0, 5, 10, 15, 20% were added into the reaction mixture to determine the effect on activity of α-1,3-glucanase HF65. Error bars represent mean values and standard deviation of triplicate determinations.

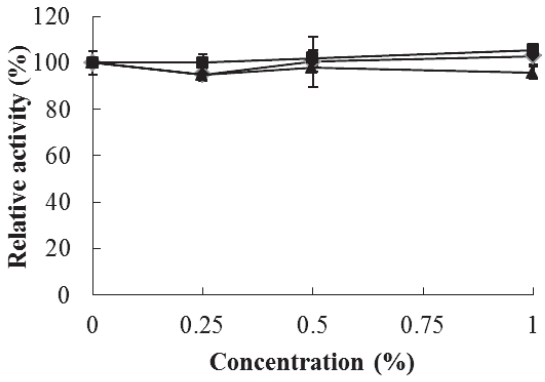

Fig. 6. Effect of the reagents, SDS (◆), NaF (■), and BTC (▲), concentrations on the activity of α-1,3-glucanase F65.

Reagents at final concentrations of 0, 0.25, 0.5, 1% were added into the reaction mixture containing 1% α-1,3-glucan, 50 mM citrate buffer (pH 5.5), and α-1,3-glucanase HF65. After incubation at 50°C for 1 h, the enzyme activities were determined using the DNS method. Error bars represent mean values and standard deviation of triplicate determinations.

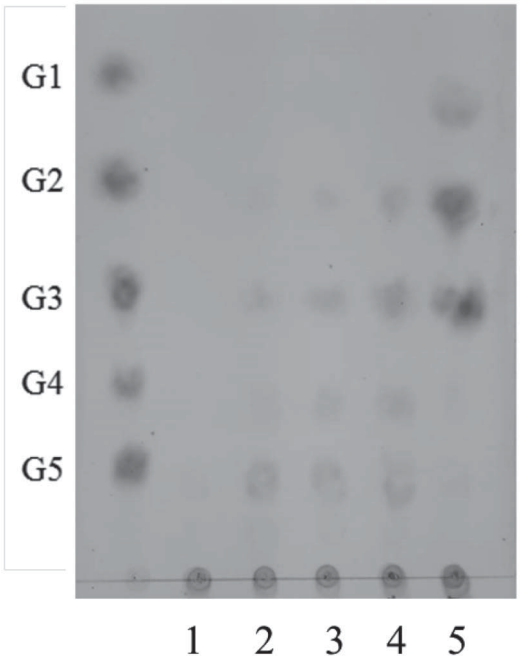

Fig. 7. TLC of hydrolysis products towards α-1,3-glucan.

TLC analysis was carried out as described in "Materials and Methods". Glucose (G1), maltrose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5) were used as molecular weight markers (lane 1). The hydrolysis products obtained after incubation for 10 min (lane 2), 30 min (lane 3), 1 h (lane 4), and 24 h (lane 5), were analyzed.

products (Fig. 7). This indicated that α-1,3-glucanase is an endo-enzyme. However, after incubation for 24 h, the enzyme released disaccharide and trisaccharide as main products and glucose as a minor product without detection of an oligosaccharide larger than tetrasaccharide.

Discussion

In this study, we selected α-1,3-glucan-assimilating bacterium *S. thermodiastaticus* HF3-3 as a source organism to produce thermostable α-1,3-glucanase. The enzyme exhibiting α-1,3-glucan-hydrolyzing activity was purified from the culture filtrate of *S. thermodiastaticus* HF3-3. A homology searching indicated that *N*-terminal amino acid sequences of the purified enzyme dose not have any similarity to those of α-1,3-glucanases reported so far. On the other hand, the sequence of eight amino acid internal residues of this enzyme was homologous with those of mycodextranases. As shown in Table 2, the amino acids in this region were highly conserved in mycodextranases, showing a high possibility that the enzyme produced by *S. thermodiastaticus* HF3-3 is grouped in mycodextranase. To date, there are only four reports for mycodextranases; *Penicillium melinii* (Tung et al., 1971), *Bacillus circuluns* NHB-1 (Okazaki et al., 1995), *Arthrobacter* sp. NHB-10 (Jin and Okazaki, 1996), and *Streptomyces* sp. J13-3 (Okazaki et al., 2001). All characterized mycodextranases were specific for α-1,4-glycosidic linkage in the α-glucan substrate consisting of alternating α-1,3- and α-1,4-linkage. Tung et al. (1971) demonstrated that mycodextranases did not hydrolyze homo α-1,3- or α-1,4-linkage substrate, suggesting that α-1,3- and α-1,4-linkages were required in the substrate for enzyme binding and cleavage (Tung et al., 1971). However, in this study, the purified enzyme clearly showed the highest specific activity toward the α-1,3-glucan substrate that only contained the α-1,3-linkage. According to these results, the enzyme purified from

S. thermodiastaticus HF3-3 should be classified in a new group of α-1,3-glucanase, designated as α-1,3-glucanase HF65 in which the amino acid sequence is highly homologous to that of mycodextranase.

The purified enzyme was also characterized in terms of its activity and biochemical properties. The optimal pH of the enzyme was 5.0–5.5, which is similar to that of α-1,3-glucanases characterized from bacteria and fungi. The enzyme was stable over an acidic pH range from 4.5 to 6.5 that was also demonstrated as a stable pH range for the other α-1,3-glucanases. Interestingly, the α-1,3-glucanase from *S. thermodiastaticus* HF3-3 was thermostable up to 65°C and also exhibited a high optimal temperature at 65°C. Almost all characterized α-1,3-glucanases exhibited maximum activity in the temperature range 45–55°C and were usually unstable at 65°C. There are only a few studies on α-1,3-glucanases that showed thermal stability at 60–65°C. Shimotsuura et al. (2008) demonstrated that mutanase RM1 of *Paenibacillus* sp. RM1 was stable at temperatures below 60°C, however it lost more than 70% of activity at 65°C after incubation for 10 min. Takehara et al. (1981) also showed that the enzyme from *Streptomyces chartreusis* F2 totally lost activity after incubation for 10 min at 65°C. This study is the first to demonstrate that α-1,3-glucanase exhibited thermotolerance at 65°C.

Some studies reported the effects of metal ions on α-1,3-glucanase activity. Generally, a significant inhibition in activity of α-1,3-glucanase by Mn^{2+} Cu^{2+} and Hg^{2+} has been reported (Imai et al., 1977; Meyer and Phaff, 1980; Sumitomo et al., 2007; Takehara et al., 1981; Wiater et al., 2013). The inhibitory effect of Zn^{2+}, Ag^{2+}, Co^{2+} and Fe^{2+} varied among α-1,3-glucanases (Meyer and Phaff, 1980; Sumitomo et al., 2007; Takehara et al., 1981). In contrast, α-1,3-glucanase HF65 in this study showed an enhanced enzyme activity with Co^{2+} and Mn^{2+}. A similar result was also observed for α-1,3-glucanase of *Paenibacillus* sp. strain KSM-M86 (Sumitomo et al., 2007). Activation of α-1,3-glucanase HF65 by Mg^{2+} ion was also in agreement with the result of α-1,3-glucanase isolated from *Trichoderma harzianum* (Wiater et al., 2013).

Many experiments have been conducted to investigate the application of α-1,3-glucanase for preventing, or removing, dental plaque *in vitro*. α-1,3-glucanase exhibited a degrading activity against *Streptococcal* biofilms (Guggenheim and Haller, 1972; Imai et al., 1977; Inoue et al., 1988; Pleszczyńska et al., 2010; Shimotsuura et al., 2008; Tsumori et al., 2011; Wiater et al., 2004, 2005). However, the practical use of the enzyme still remains uncertain. The stability of α-1,3-glucanase in the common ingredients of oral care products has rarely been described. In contrast, dextranase, which hydrolyzes α-1,6-glucosidic bonds in dental plaque, has been used as an effective agent in commercial toothpaste (Lion Corporation, Japan). There are many reports on the stability and activity of dextranase with effective mouthwash ingredients such as sodium benzoate (0.02% w/v), NaF (0.05%), and SDS (0.05% w/v) (Kim et al., 1999; Marotta et al., 2002; Wang et al., 2014). In this study, desirable properties of α-1,3-glucanase HF65 for application in oral care products have been reported. The enzyme could be stable in SDS, BTC, and NaF up to the 1% w/v concentration used in commercal products. Moreover, the enzyme retained full activity even in the presence of NaCl (20% w/v) and an increased activity to 130% of the initial activity at 5% NaCl. These results indicate the potential use of α-1,3-glucanase HF65 in dental care products, including saline mouthwash and toothpaste.

In conclusion, we have found a new source to produce α-1,3-glucanase. The enzyme was stable at 65°C and could tolerate a high concentration of salt and surfactants. As mentioned in the Introduction, many α-1,3-glucanases exhibited their efficiency to reduce the dental plague produced by *Streptococci in vitro*. However, the actual use of the enzyme still faces some difficulties due to its reduced stability. In this study, we characterized α-1,3-glucanase HF65 from *S. thermodiastaticus* HF3-3 and showed a high potential of the enzyme as a candidate for commercial use based on its properties. Its gene structure and the relation between its function and structure remain to be clarified in the future.

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