Agric. Chem. Biotechnol. **43**(4), 246-249(2000)

Articles

The Degradation of Chitin with Food Grade Papain

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Received June 2, 2000

We investigated the production of chitin oligosaccharides using food grade papain. A solution of commercial food grade papain (FGP) was dialyzed for 12 h before measuring its chitinolytic activity. The effects of enzyme concentration, reaction temperature, and pH on the endochitinase and β-*N*-acetylglucosaminidase activities and the thermostability of these enzymes were investigated. In adddition, the reaction products were analyzed with gel filtration on a Bio-Gel P2. The endochitinase activity was twentyfold higher than that of β-*N*-acetylglucosaminidase. The optimal endochitinase activity was at pH 3.0, while the maximal β-*N*-acetylglucosaminidase activity was at pH 6.0. The reaction product consisted mainly of the dimer of *N*-acetylglucosamine, with a small amount of its trimer. Under the experimental conditions, 120 μg of chitin oligomers were obtained with 1 mg of FGP protein after an incubation of 2 h.

Key words: *chitin oligomers, food grade papain, β-N-acetylglucosaminidase, endochitinase.*

Chitin, a polymer of *N*-acetylglucosamine(GlcNAc) in β(1→4)glycosidic linkage with a molecular weight in the several millions, is the most abundant polysaccharide, next to cellulose. Since it is minimally soluble in water, organic solvents, and dilute acids or bases, its use is strictly limited.[1,2] However, chitin and chitosan oligosaccharides are reported to have high potential as food ingradients and medico-pharmaceuticals, including immune stimulants.[3,4] For its industrial application, chitinase has been investigated. Chitinases are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive GlcNAcs of chitin. These chitinases consist of two hydrolases. The first one is an endochitinase (EC 3.2.1.14), which splits chitin into oligomers of GlcNAc. The second one is a β-*N*-acetylglucosaminidase (EC 3.2.1.30) hydrolyzes oligomers into free GlcNAc. On the other hand lysozyme cleaves a bond between the C1 of *N*-acetylmuramic acid and the C4 of GlcNAc in the bacterial peptidoglycan.[5]

Papain, as well as latex from the fig and hevea is known to contain considerable chitinase activity which have been purified and well characterized.[6-11] However, the complex purification procedures involved result in low yields, sufficient only for analytical studies. In order to produce chitin oligosaccharides, inexpensive commercial chitinases must be available. Although several studies have investigated the utilization of papain for the production of chitosan oligomers,[12-14] the production of chitin oligosaccharides through enzymatic hydrolysis have not been well-studied. In this paper, we report on the optimal conditions for the production of chitin oligomers with commercially available food grade papain (FGP).

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Abbreviations: FGP, food grade papain; GlcNAc, *N*-acetylglucosamine.

Materials and Methods

Materials. FGP (Collupulin®, Gist Brocades, France) was purchased from a local supplier. Snail β-glucuronidase (Sigma, USA) was diluted in the incubation buffer before use. *N*-acetylglucosamine (Sigma, USA) and casein (Merck, Germany) were bought from local dealers. The standard chitin oligomers (dimer, trimer, pentamer, and hexamer) were supplied by Seikagaku Co. (Japan). All other reagents were of analytical grade.

Assay of papain chitinolytic activity. Food grade papain (Collupulin®) was dissolved in citrate-phosphate buffer (pH 5.2, 40 mM) and dialyzed for at least 12 h before use. The β-*N*-acetylglucosaminidase and endochitinase activities were measured as previously described.[15] The β-*N*-acetylglucosaminidase assay was carried out by adding an enzyme solution containing 1 mg of protein to 2 mg of colloidal chitin in 4 m*l* of 40 mM citrate/Na_2HPO_4 buffer, pH 5.2 at 50°C for 2 h. The reaction mixture was boiled for 10 min and centrifuged at 3,000 g for 20 min. The supernatant was used to measure the β-*N*-acetylglucosaminidase activity through colorimetric determination of GlcNAc with ρ-dimetylaminobenzaldehyde.[16] The preparation of [^{3}H]-chitin and subsequent measurement of chitinase activity were performed using the method described by Molano *et al.*[17] To measure the endochitinase activity, 500 μ*l* of the supernatant, previously used to measure the β-*N*-

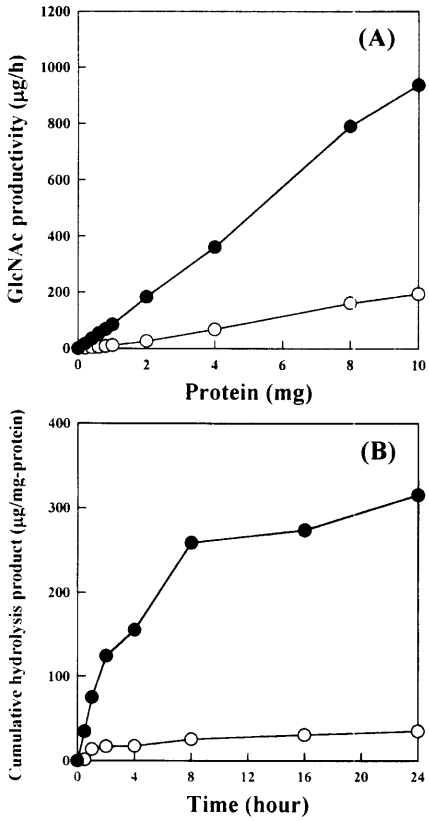

Fig. 1. The concentration (A) and incubation time (B) on the chitinolytic activity of food grade papain. (○), β-N-acetylglucosaminidase activity; (●), endochitinase activity. (A) The reaction was carried out at 50°C for 2 h. (B) The reaction was carried out with 1 mg of protein at 50°C and pH 5.2.

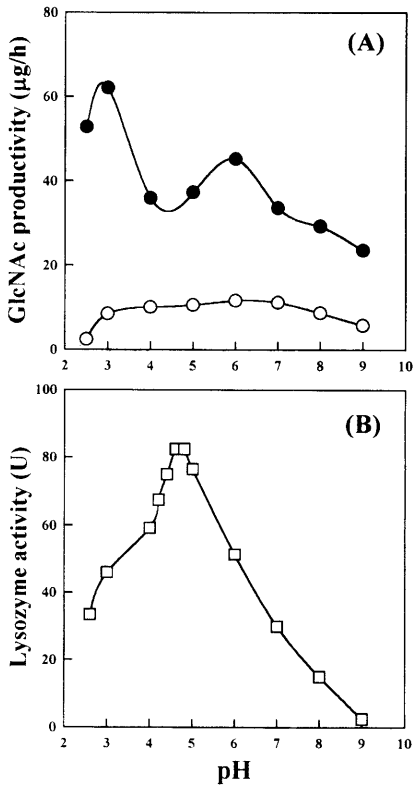

Fig. 2. Effect of pH on the chitinolytic (A) and lysozymal (B) activities of food grade papain. (○), β-N-acetylglucosaminidase activity; (●), endochitinase activity. The reaction was carried out with 1 mg of protein at 50°C for 2 h. The reaction was carried out with 1mg of protein at 37°C for 1 h.

acetylglucosaminidase, was mixed with 100 μl of snail gut β-glucuronidase and kept at 37°C for 1 h, during which the β-glucuronidase completely converted the water-soluble oligomers into GlcNAc. The difference in the activities before and after the treatment with β-glucuronidase was taken to be the activity of the endochitinase. Protein content was measured using the method of Lowry *et al.*[18]

Assay of papain proteolytic and lysozymal activity. The proteolytic activity assay of papain was performed with casein using the method reported by Yamaguchi *et al.*[19] One unit is defined as the amount of enzyme needed to diminish the optical density at 280 nm by 0.001 at 40°C for 1 min. The lysozymal activity was measured with the cell wall of *Micrococcus lysodeikticus* using the method of Martin.[20]

Optimizing the reaction conditions for chitinolytic activity. To find the optimal conditions for papain, the concentration of papain was increased up to 10 mg, and the reaction temperature was varied from 30 to 90°C in 10°C increments. The effect of pH on lysozyme activity was also investigated. Citrate-phosphate buffer was employed to obtain pH values from 2.5 to 7.0, and phosphate buffer for pH values from 7.0 to 9.0.

Thermostability of the hydrolytic activities of papain. To determine the thermostability of chitinolytic, proteolytic, and lysozymal activities, the enzyme solution was kept at 50, 60, and 70°C for periods of 1 to 3 days. The activities were measured as described above.

Analysis of reaction products. Gel filtration chromatography was performed on a column (1×150 cm) filled with Bio-Gel P2 (BioRad, USA). After adding 2 ml of the reaction mixture, the column was eluted with distilled water at 0.6 ml/min, and 2 ml fractions were collected. Using [^{3}H]-labeled chitin as the substrate, the radioactivity was measured with a liquid scintillation counter (TR 1600 Packard, USA).

Results and Discussion

Optimization of reaction conditions. The formation of chitin oligomers increased linearly with the concentration of papain utilized up to 10 mg (Fig. 1A). The activity of endochitinase was clearly 6-7 times higher than that of β-*N*-acetylglucosaminidase (Fig. 1A). This result is in good accord with the report of Howard and Glazer,[9] in which the enzyme purified from papain hydrolyzed less than 1% of the added substrate by the action of β-*N*-acetylglucosaminidase. The effect of reaction time on chitinolysis by papain is shown in Fig. 1B. The formation of product increased

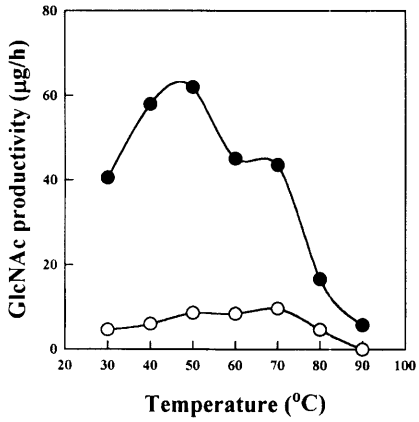

Fig. 3. Effect of temperature on the chitinolytic activity of food grade papain. The reaction was carried out with 1 mg of protein at pH 5.2 for 2 h. (○), β-*N*-acetylglucosaminidase activity; (●), endochitinase activity.

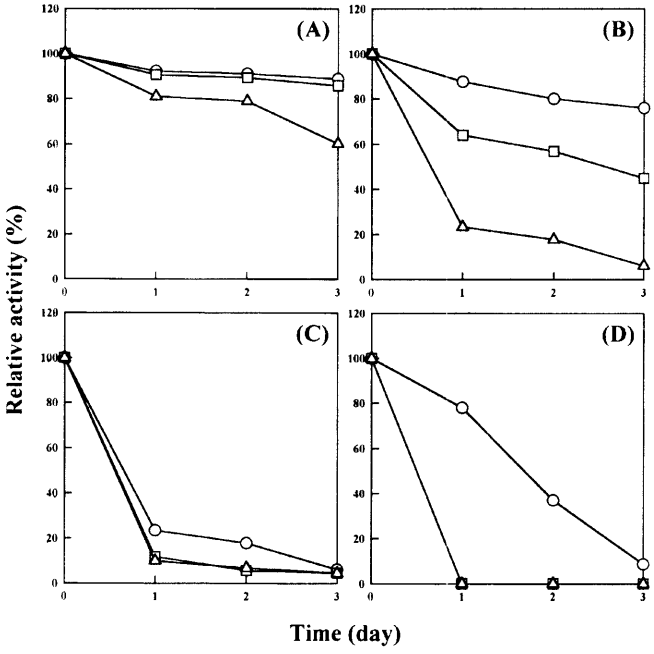

Fig. 4. Thermostability of the hydrolytic activities of food grade papain. (A) Endochitinase, (B) β-*N*-acetylglucosaminidase, (C) Proteolytic, and (D) Lysozymal activities. (○), 50 °C; (□), 60 °C; (△), 70 °C.

linearly with reaction time up to 2 h. After 8 h of incubation, the formation of chitin oligomers reached a plateau of 260 μg/mg-protein, and thereafter no significant increase in formation was observed. With 10 mg of papain, 1.4 mg of chitin oligomers was obtained after a 2-h incubation (Fig. 1B).

The effect of pH on the endochitinase and β-*N*-acetylglucosaminidase activities is shown in Fig. 2A. The endochitinase activity was maximal at pH 3, but also had a second optimal pH at 6.0. Even at pH 9.0, endochitinase retained 40% of its maximal activity. In contrast, the optimal pH for β-*N*-acetylglucosaminidase was between 4 and 7

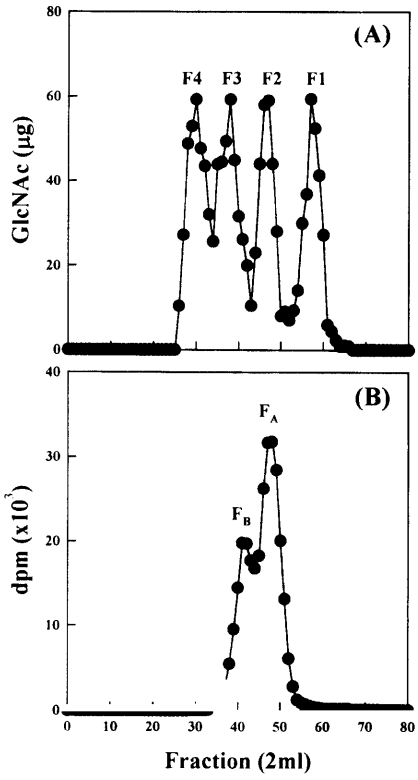

Fig. 5. Separation of chitin oligomers on Bio-Gel P-2 column. Separation of chitin oligomers: F1, GlcNAc; F2, dimer of GlcNAc; F3, trimer of GlcNAc; F4, pentamer of GlcNAc. Chitin oligomers formed by food grade papain.

(Fig. 2A). It is generally accepted that the optima pH of plant chitinases are acidic.[20,21] When visualized with activity staining after gel electrophoresis, more than 4 bands were observed on the gel (unpublished data). Multiple activity bands and two optimal pH may suggest that the FGP is a mixture of several chitinases. On the other hand, the lysozymal activity of papain had optimal pH around 5.0 (Fig. 2B) and almost completely lost its activity at pH 9.0.

The effect of reaction temperature on the chitinolytic activity of papain is shown in Fig. 3. The activity of β-*N*-acetylglucosaminidase increased slightly up to 70°C and fell to the level of 30°C at 80°C. However, the optimal temperature of endochitinase was 50°C, and 70% of its activity remained at 70°C. Subsequently, it decreased rapidly to the 30% level at 80°C.

Based on these results, we determined that the optimal conditions for chitinolysis by papain are incubation with 1 mg of papain protein, at pH 3.0 and at 50°C for 2 h. Under these conditions, 120-130 μg of chitin oligomers per mg protein of papain were produced in 2 h.

Thermorstability of the hydrolytic enzyme activity of papain. The thermostability of the chitinolytic, lysozymal, and proteolytic activities of papain is shown in Fig. 4. The endochitinase activity was stable at 50 and 60°C for 3 days, and 80% of the activity remained when kept at 70°C for 2 days (Fig. 4A). As seen in Fig. 4B, the β-*N*-acetyl-

glucosaminidase activity is less stable than endochitinase activity. Even after one day at 70°C, the β-*N*-acetyl-glucosaminidase activity diminished to 20% level (Fig. 4B). This thermostability of the endochitinase activity was in good agreement with the result shown in Fig. 3. In contrast, the proteolytic activity of papain rapidly diminished to 10-15% level, even after one day, at all temperatures (Fig. 4C). In addition, at 50°C the lysozyme activity of papain was more than the proteolytic activity, but at 60°C and 70°C no activity was detected (Fig. 4D).

Analysis of the reaction products by gel filteration. *N*-acetylglucosamine (GlcNAc) and its dimer, trimer, and pentamer were applied as standards on the Bio-Gel P2. The elution profile of these standards and the reaction products of [^{3}H]-labeled chitin are shown in Fig. 5. The reaction mixture was composed mainly of the GlcNAc dimer, with a small portion of the trimer. Howard and Glazer[9] reported that the dimer was obtained when the tetramer of GlcNAc was incubated with the purified enzyme. Although different substrates and enzymes were utilized in the assay, it should be noted that the GlcNAc dimer was the major product formed by papain. Furthermore, gel filtration using water as the elution solvent is advantageous for obtaining purified products.

Acknowledgments. This work was partially supported by the research grant from Agricultural Research and Promotion Center, Ministry of Agriculture and Forestry.

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