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Characteristics of galactomannanase for degrading konjac gel

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Abstract

Galactomannanase (Glmnase) is an enzyme product derived from *Aspergillus niger*. The activity of Glmnase degrading (hydrolyzing) the konjac gel were investigated. Significant loss in the enzyme activity was found when the temperature above 60 °C. Similar observations were obtained when the reaction pH above 5. Further increase in the pH value resulted in entirely loss of enzyme activity at the alkaline pH region (pH 8.0 and above). The optimal hydrolyzing temperature and pH were at 60 °C and 5.0, respectively. For the stability test, the purified Glmnase increased its thermostability up to 70 °C at pH 5.0, but it retained only about 60% activity after 60 min incubation at this temperature and its activity became zero after 20 min incubation at 80 °C. The Glmnase was stable at the pH range from 3.0 to 7.0 at room temperature and retained at least 80% activity for 60 min. For the storage temperature test, the lyophilized Glmnase still conserved about 90% activity during 7 days at 30 °C, and was higher than about 80% at 4 °C. The K_m and V_{max}, were 0.018 mg/ml konjac powder and 0.20 mg/ml reducing sugar per min, respectively.

Keywords: Galactomannanase; Konjac; Enzyme; Activity; Stability

1. Introduction

Konjac glucomannan (KGM) is a high molecular weight, water-soluble and non-ionic (neutral) polysaccharide found in roots and tubers of the *Amorphophallus konjac* plant. It has D-mannose (M) and D-glucose (G) in M/G molar ratio of 1.5–1.6 by β-1,4-glycosidic linkages with about 1 acetyl group in every 17–19 sugar units at C-6 position [1]. KGM has long been used in TCM (tradition chinese medicines) as an immunoregulating and health-care food, such as an indigestible dietary fiber, beneficial for digestion system. Oligosaccharides have been reported to play important roles in biological system like the health diet system of humans and farm animals by the growth of bifidobacteria and by affecting more subtly the immunology status of intestinal cell [2]. Recent study demonstrates that the function of Konjac oligosaccharide (KOS) on a diabetes model of isolated islet was evaluated and the results show one component of KOS consisted of tetrasaccharide could regulate $NO^{\bullet}$ level on streptozocin (STZ)-treated islets in a dose-dependent manner [3].

In general, the most practical and useful method to produce KOS is enzymatic degradation of KGM employing the relevant endo-enzymes, e.g. β-mannanase (1,4-β-D-mannan mannohydrolase, EC3.2.1.78). The β-mannanase can hydrolyze 1,4-β-D-mannopyranosyl linkages of polysaccharides (e.g. konjac powder, locust bean gum, guar gum, etc.), such as mannans, glucomannans, galactomannans and galactoglucomannans, yielding manno-oligosaccharides. This enzyme has been isolated and characterized from microbial sources [4–10]. The galactomannanase (1,4-β-D-galactan-4-mannano-hydrolase, Glmnase) is an enzyme product derived from *Aspergillus niger*. Glmnase, a hemicellulose-degrading and water-soluble enzyme, is generally used to remove the sediment from coffee extracts, to peel soybean and to be an anti-staling agent (especially for the presence of small amount of glucomannan or galactomannan like guar gum) in food industry. It is also used to desize fabrics made from synthetic fibers in textile industry. The interests in β-mannanase and other hemicellulose-degrading enzymes, e.g. Glmnase, have recently increased, partly because of their potential applications in the food, textile and paper or pulp industries

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[7,11]. However, there is no information so far available about Glmnase from *A. niger* for KGM hydrolysis. In the present study, the aim was to achieve gel hydrolysis of konjac powder by Glmnase from *A. niger* and to report some enzymatic properties.

2. Materials and methods

2.1. Materials

The crude Glmnase from *A. niger* was a gift from President C.K. Lin (Challenge Bioproducts Co., Ltd., Yun-Lin Hsien, Taiwan) and further purified by gel filtration chromatography (Bio-Gel P-30, Bio-Rad Laboratories, Inc., Hercules, CA). The partially purified Glmnase solution was dialyzed at 4 °C overnight in deionized water and then lyophilized. Konjac powder (glucomannan content ≥79%, (w/w)) was purchased from Asahi-Ya Food Co., Ltd (Taipei Hsien, Taiwan). Konjac gel was prepared by heat-gelatinization, modified from that reported by Lin and Huang [12], of 4%, (w/v), konjac powder (flour) in 0.5% sodium carbonate (pH 11.5) and autoclaved for 30 min. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Molecular mass of Glmnase

For SDS-PAGE, the Glmnase was dissolved in sample buffer (0.125 M Tris–HCl buffer, pH 6.8, containing 20% glycerol, 3 μM bromophenol blue, and 1% β-mercaptoethanol) and heated at 100 °C for 5 min. The electrophoresis was conducted by the Laemmli method [13] with a 10 μl sample on the 12% polyacrylamide gel using a vertical slab electrophoresis apparatus (Mini-Protein III, Bio-Rad Laboratories, Inc., Hercules, CA). After the electrophoresis (∼2 h), the gel was stained with Coomassie blue G. For the gel filtration chromatography, the sample was loaded to a Bio-Gel P-30 size exclusion matrix and eluted with 50 mM phosphate buffer (pH 5.0) applied to the column at flow rate of 0.25 ml/min. The absorption of the effluent of the crude Glmnase solution at 280 nm was measured by a UV–vis spectrophotometer (Helios Alpha, Unicam Instruments, Cambridge, UK), while the 28 kDa ovomucoid and 45 kDa ovalbumin were used as standards for molecular mass determination.

2.2.2. Enzyme assay of Glmnase

The reaction mixture for the Glmnase assay containing 1.0 g konjac gel in 2.5 ml of 50 mM phosphate buffer (pH 7.0) and 1.5 ml of suitably diluted enzyme solution (1.0% (w/v) of Glmnase) was incubated at 60 °C for 30 min, and then 1.0 ml of the reaction mixture was withdrawn and heated to 100 °C for 10 min to inactivate the enzyme prior to reducing sugar measurement. The amount of reducing sugar liberated was defined by subtraction of the amount before incubation at time zero from that after incubation at 60 °C for 30 min. The amount of reducing sugar was determined by dinitrosalicylic acid (DNS) colorimetric method. One unit of Glmnase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalent to glucose per minute at 60 °C.

2.2.3. Effects of reaction temperature and pH on Glmnase activity

The reaction mixture (see above) was incubated at different temperature (30–80 °C) for 30 min. The Glmnase activity was assayed under the above condition. The pH dependence of the Glmnase activity was examined within the range pH 3–8 (pH 3–6: 50 mM citrate–phosphate buffer; pH 7–8: 50 mM phosphate buffer). Activity at each pH value was determined as described above.

2.2.4. Effects of incubation temperature and pH on Glmnase stability

The Glmnase (3.75 μg/ml) was incubated in 50 mM citrate–phosphate buffer (pH 5.0) at different incubation temperatures for 60 min. Following incubation, the remaining activities were measured as described above. Similarly, the purified Glmnase at different pH values (pH 3–8) was incubated at room temperature for 60 min. The pH optimum was determined by following incubation and measurement of the remaining activities as described above.

2.2.5. Effect of storage temperature on Glmnase stability

For enzyme storage stability test, the lyophilized purified Glmnase was stored at 4 and 30 °C for 7-day storage test of the remaining enzyme activity by following measurement of the activity as described above.

2.2.6. Determination of kinetic parameters for Glmnase

The initial reaction rate (v_0) in the presence of various substrate concentrations was determined. Glmnase was determined by reducing sugar measurement of enzyme activity with various concentrations of konjac powder at 60 °C and pH 5.0 (50 mM citrate–phosphate buffer) for 30 min. The values of K_m and V_{max}, were determined from a Lineweaver–Burk plot.

3. Results and discussion

3.1. Molecular mass of Glmnase

The data from the loaded sample (crude Glmnase) as assayed by SDS-PAGE showed two major proteins, one of about 83 kDa and one of about 45 kDa, one minor protein of about 36 kDa (Fig. 1 inset). Further purification by Bio-Gel P-30 gel filtration chromatography yielded the partially purified enzyme (about 45 kDa) preparation (Fig. 1). The Glmnase activity could be tested by hydrolyzing guar gum powder through the activity factor (AF), which is defined as $AF = 2/(T \times A)$, where T is the time in minutes required for the dough to start flowing and A is the absorption of the effluent at 280 nm (data not shown). This suggests that the enzyme of about 45 kDa is more likely to be Glmnase rather than that of about 36 kDa, leading to the conclusion that it is probably capable of providing the degradation effect on konjac gel.

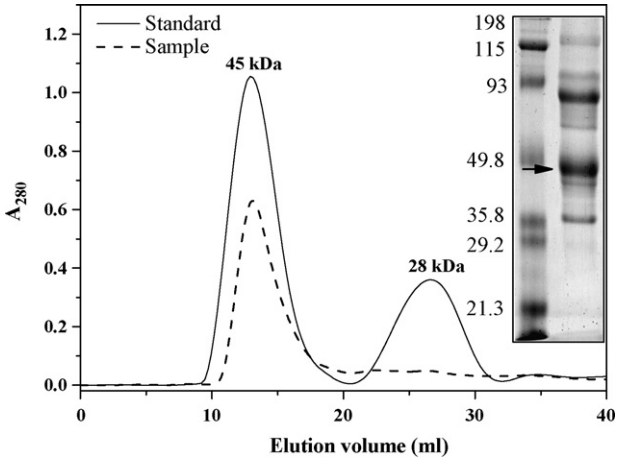


Fig. 1. Gel filtration chromatography of the Glmnase on a 32 ml Bio-Gel P-30 gel column in 0.3% NaCl solution. Inset: SDS-PAGE (12%) electrophoresis of the crude Glmnase enzyme from *A. niger* (right lane: maker; left lane: sample). The band showed at 45 kDa (as point at arrow) is more likely the Glmanase. This is coincided with the observation in the previous size exclusion chromatography.

3.2. Optimal temperature and pH on Glmnase activity and stability

The partially purified Glmnase activity of hydrolyzing konjac gel was found by varying the reaction temperature. The maximal activity was centered at 60 °C under pH 7.0 (Fig. 2A). Partially purified Glmnase maintained a higher activity between 30 and 60 °C. Yet, the Glmnase displayed the highest activity at 60 °C, and then progressively decreased at higher temperature (Fig. 2A). On the other hand, the optimal pH for the hydrolyzing activity of the partially purified Glmnase was 5.0 at 60 °C. The Glmnase was proven to be stable in the reaction pH range of 3.0–6.0, while a totally loss in the Glmnase activity was found at reaction pH above 8.0 (Fig. 2B). This result may be due to the acidity of Glmnase, which was similar to that of acidic β-mannanase from *Sclerotium rolfsii* [16]. As a result, the optimal reaction temperature and pH for Glmnase activity was at 60 °C and 5.0, respectively.

The 45 kDa Glmnase, as used in this study, was very similar to that of β-mannanase from *A. niger*, which has been proven to be stable in the pH range 3–8 and below 70 °C. The pH optimum has been determined to be 3.0 as reported by McCleary [14,15]. Moreover, the optimum activity of the crude Glmnase for hydrolysis of locus bean gum has been found at pH 4.0 and 75 °C and proved to be stable in the pH range 3–9, as stated by President C.K. Lin.

Stability of the Glmnase activity, as a function of time (minutes) of incubation at various temperature (from 30 to 80 °C) and pH values (from pH 3 to 8), is shown in Fig. 3. From Fig. 3A, it can be seen that the thermal stability of Glmnase activity decreased as the incubation time in the temperature interval of 30–80 °C increased. Glmnase could maintain the thermostability up to 60 °C, almost 80% of the relative activity was retained for 60 min incubation at this temperature. However, the Glmnase activity became zero after 20 min incubation at 80 °C. This result indicated that Glmnase was not the thermostable component at

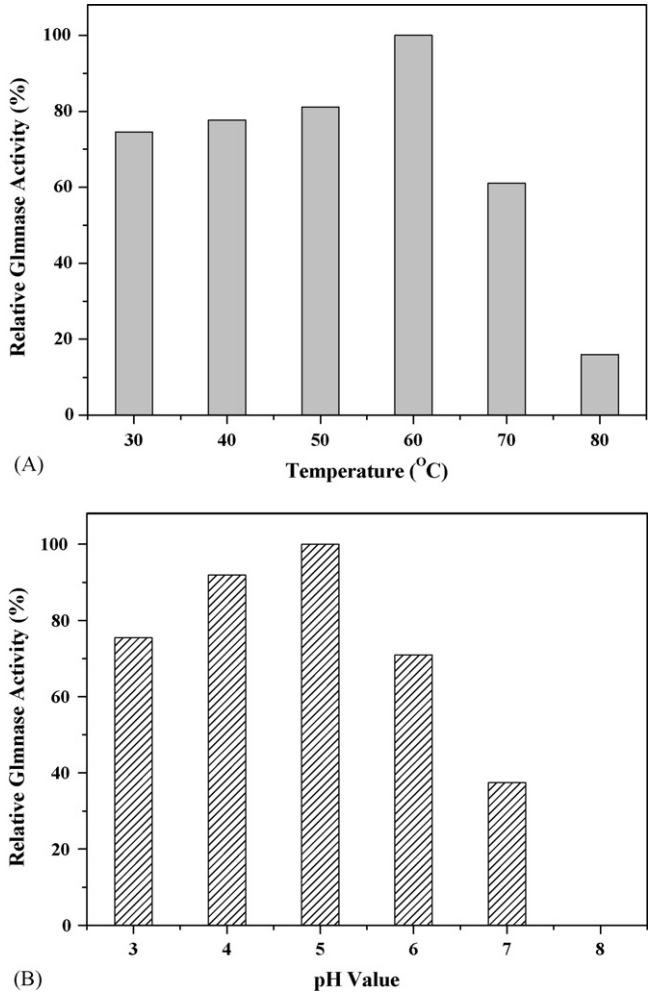
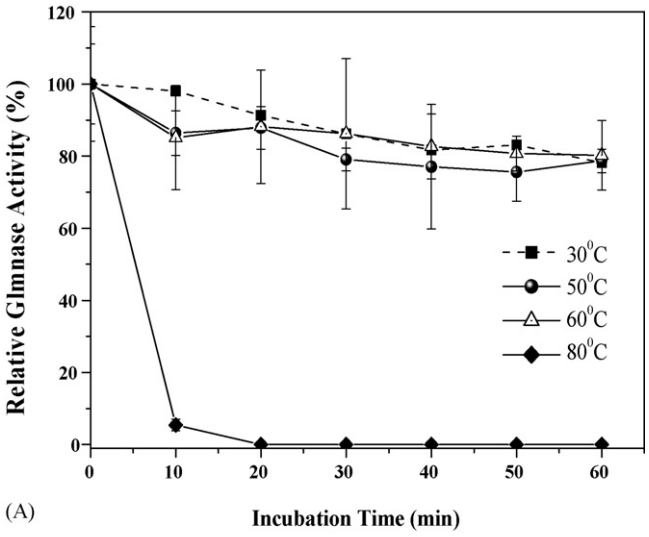


Fig. 2. (A) Effect of reaction temperature on the purified Glmnase (3.75 μg/ml) activity at pH 7.0 for 30 min. 100% value represents 105.0 units. (B) Effect of reaction pH on the purified Glmnase (3.75 μg/ml) activity at 60 °C for 30 min (pH 3–6: 50 mM citrate–phosphate buffer; pH 7–8: 50 mM phosphate buffer).

80 °C or higher temperature. Fig. 3B shows the pH stability of the partial Glmnase. It can be seen from this figure that over 80% of Glmnase activity was still retained between pH 3.0 and 7.0 for 60 min under experimental condition. The remaining activity of Glmnase, however, was decreased as the incubation time at pH 8.0 (Fig. 3B) or higher (data not shown) increased. These results together suggest that the Glmnase appeared to be more stable at acidic and neutral pH (pH ≤7.0) than at alkaline pH (pH > 7.0) at room temperature. The result may also reflect to the acidity of this enzyme.

3.3. Effect of storage temperature on Glmnase stability

The result of storage stability of Glmnase was shown in Fig. 4. As shown in this figure, the lyophilized partially purified Glmase was very stable at 30 °C and retained at least about 90% of its activity after being stored for 7 days at this temperature. Similar results were also obtained at 4 °C where its relative activity remained at about 80% level after 7 day of storage (Fig. 4). This



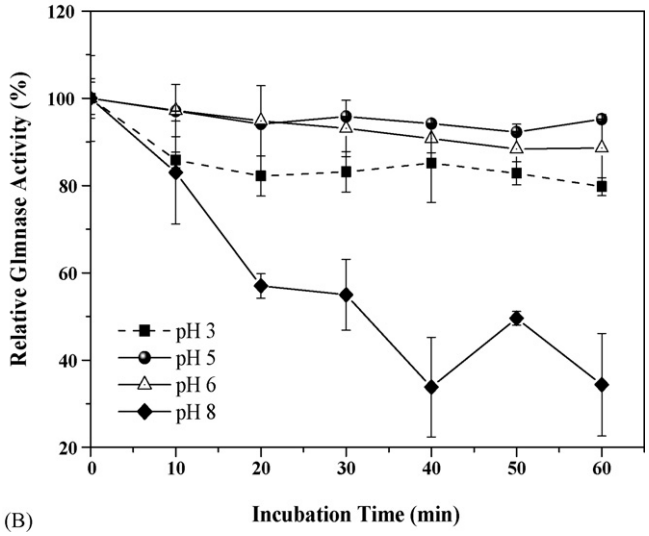


Fig. 3. (A) Effects of incubation temperature and time on the partially purified Glmnase stability at pH 5.0. (B) Effects of incubation pH and time on the purified Glmnase stability at room temperature (pH 3–6: 50 mM citrate–phosphate buffer; pH 7–8: 50 mM phosphate buffer).

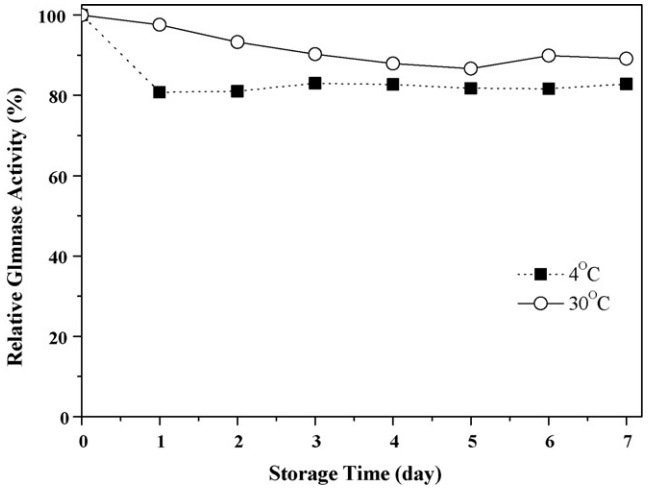


Fig. 4. Effects of storage temperature and time on the lyophilized Glmnase stability.

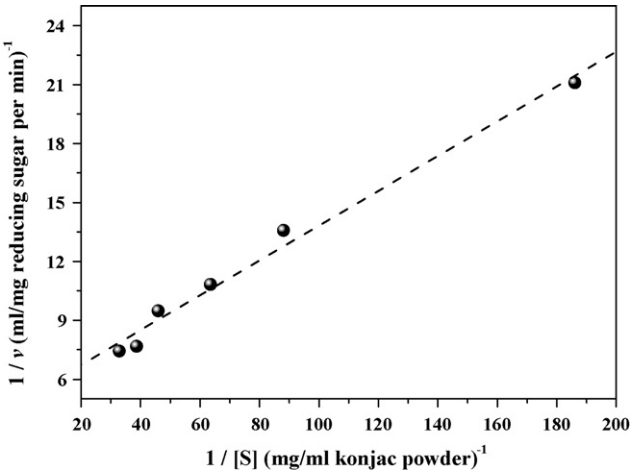


Fig. 5. Lineweaver–Burk plot for the purified Glmnase acting on the gel of konjac powder.

result indicated that the lyophilized Glmnase appear to be very stable for a period of time.

3.4. Kinetic parameters for Glmnase

The initial reaction rate for releasing reducing sugar from the gel of konjac powder governed by Glmnase was determined at various concentrations of konjac powder (as substrate) under the assay conditions (60 °C, 30 min). The Lineweave–Burk plot of the Glmnase acting on the gel of konjac powder was presented in Fig. 5. The values of K_m and V_{max}, calculated from the slope and intercept of the plot, for konjac powder were 0.018 mg/ml konjac powder and 0.20 mg/ml reducing sugar per min, respectively under the experimental conditions. Additionally, the K_m of 0.0018% (w/v) for Glmnase acting on KGM, and 0.096% (w/v) for β-mannanase from *A. niger* acting upon *Orchis intacta* glucomannan were observed [17]. These different results may be due to the difference of enzyme and substrate specificity.

4. Conclusion

In this study, the optimum activity of the purified Glmnase acting on the gel of konjac powder has been found at pH 5.0 and 60 °C and proved to be stable in the pH range 3–6 at room temperature. The enzyme was stable in the range of 30–70 °C when it was incubated at 30–80 °C for 30 min to 2 h. Nevertheless, the enzyme activity was nearly lost after 15 min at 80 °C. In fact, the complete enzymatic hydrolysis of locust bean gum galactomannan to oligomers of galactose and mannose requires the action of β-mannanase, α-galactosidase and β-mannosidase, which were both produced mainly from *A. niger* [8]. However, the hemicellulase Glmnase of 45 kDa enzyme produced from *A. niger* employed in this study was similar to cellulase and β-mannanase [18], which was probably an important contributor to degrade KGM for KOS of glucose and mannose. It seems important to investigate the detailed action of Glmnase on glucomannan hydrolysis and the use of the other synergistic enzymes for extending its potential.

Acknowledgements

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