Enzymatic Hydrolysis of Mannan from Konjac (*Amorphophallus sp.*) Using Mannanase from *Streptomyces lipmanii* to Produce Manno-oligosaccharides

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Abstract

Mannan is an abundant polysaccharide that can be found in konjac (*Amorphophallus* sp.). Mannan can be enzymatically hydrolyzed using mannanase to produce manno-oligosaccharides which can be used as a prebiotic. The aims of this research are to determine the production time of mannanase from *Streptomyces lipmanii*, perform enzyme characterization, optimize the hydrolysis time, and characterize the hydrolysis product. A qualitative assay using the indicator Congo red showed that *S. lipmanii* generated a clear zone, indicating that *S. lipmanii* produced mannanase in konjac medium and possessed mannanolytic activity. Enzyme activity was determined through reducing sugar measurement using the dinitrosalycylic acid method, and optimum enzyme production was achieved at the second day of culture. Characterization of the enzyme showed that hydrolysis was optimum at pH 7 and at a temperature of 50 °C. The reducing sugar content was increased by an increasing the hydrolysis time, and reached an optimum time at 2 h. The degree of polymerization value of three was achieved after 2 h hydrolysis of mannan from konjac, indicating the formation of oligosaccharides. Analysis by thin layer chromatography using butanol, acetic acid, and water in a ratio of 2:1:1 as eluent showed the presence of compounds with a retention time between those of mannose and mannotetrose. Confirmation was also performed by HPLC, based on the retention time.

Abstrak

Hidrolisis Enzimatik Mannan dari Umbi Porang (*Amorphophallus* sp.) menggunakan Enzim Mannanase dari *Streptomyces lipmanii* untuk Pembuatan Manno-oligosakarida. Mannan adalah polisakarida yang melimpah yang dapat ditemukan pada umbi porang (*Amorphophallus* sp.). Mannan dapat dihidrolisis secara enzimatik menggunakan mannanase untuk memproduksi manno-oligosakarida, yang dapat digunakan sebagai prebiotik. Tujuan dari penelitian ini adalah menentukan waktu produksi mannanase dari *Streptomyces lipmanii*, karakterisasi enzim, optimasi waktu hidrolisis, dan karakterisasi produk hidrolisis. Uji kualitatif dengan menggunakan *Congo red*, menunjukkan bahwa *S. lipmanii* menghasilkan zona bening, yang menunjukkan bahwa *S. lipmanii* menghasilkan mannanase dalam medium umbi porang dan memiliki aktivitas mannanolitik. Aktivitas enzim ditentukan melalui pengukuran gula pereduksi menggunakan metode asam dinitrosalisilat, dan produksi optimum enzim dicapai pada kultur hari kedua. Karakterisasi enzim menunjukkan bahwa reaksi hidrolisis optimum pada pH 7 dan suhu 50 °C. Kadar gula pereduksi meningkat dengan bertambahnya waktu hidrolisis, dan mencapai optimum pada jam kedua. Derajat polimerisasi senilai 3 telah tercapai setelah hidrolisis mannan selama 2 jam yang menunjukkan terbentuknya oligosakarida. Analisis dengan kromatografi lapis tipis menggunakan eluen butanol, asam asetat, dan air dengan rasio 2:1:1, menunjukkan adanya senyawa yang memiliki faktor retensi antara mannosa dan mannotetrosa. Hasil tersebut juga dikonfirmasi dengan kromatografi cair kinerja tinggi, berdasarkan waktu retensi senyawa.

Keywords: hydrolysis, mannanase, manno-oligosaccharide, konjac, Streptomyces lipmanii

Introduction

Formulated milk has recently become a topic of discussion due to some cases of concern, for example, the discovery of bacteria in the product. To overcome this issue, innovations have been introduced, one of which is the addition of a prebiotic [1]. A prebiotic is a food supplement that consists of non-digestible carbohydrates that induce

the growth of beneficial bacteria in the digestive system of the host [2].

Many research institutions as well as commercial companies work on the development of prebiotics, most of which have been in the form of oligosaccharides [3]. Oligosaccharides can be obtained directly from several types of plants, such as dates and dragon fruit [4], and are indirectly derived from the hydrolysis of polysaccharides. Polysaccharides can be chemically or enzymatically hydrolyzed into oligosaccharides and monosaccharides [5].

Polysaccharides used in the production of oligosaccharides are generally obtained from the remains of processed products or leftovers, such as coffee extract residue, copra, konjac (*Amorphophallus* sp.) and palm kernel cake; hence its cost is relatively low. Some of these products are listed as ingredients in packaged food.

Konjac (*Amorphophallus* sp.) is a plant from the *Araceae* family that grows only in the tropics and subtropics. In Indonesia, this plant is not yet widely cultivated and grows in the wild. Konjac contains 25-55%, depending on the environmental conditions and growth phase, of the polysaccharide glucomannan [6]. Glucomannan levels in konjac can reach up to 70% [7]. Mannan polysaccharides could be hydrolyzed to oligosaccharides or monosaccharides through the synergistic activity of β-mannanase (mannan endo-1,4-β-mannosidase; EC 3.2.1.78) and β-mannosidase (exo-β-mannosidase; EC 3.2.1.25).

Endo-1,4-β-mannanase, often referred to as β-mannanase, is a type of enzyme hemicellulase. Norita et al. [8] reported that β-mannanase produced by *Aspergillus niger* has optimum activity at pH 3.5, while β-mannanase produced by *Scopulariopsis candida* is more active at pH 6. Mannanase activity is optimum at an incubation time of 4-6 h. Most of the β-mannanase degrades mannan to a degree of polymerization 4, and sometimes mannotriose is also produced from the hydrolysis process. Hydrolysis by β-mannanase derived from fungi and bacteria produces mannohexose. Yopi *et al.* [5] reported that the activity of β-mannanase from *Streptomyces lipmanii* in palm kernel cake is 0.032 U mL^{-1}.

Based on the monosaccharide unit, mannan polysaccharides are classified into three groups: mannan, galactomannan, and glucomannan [9]. Polysaccharide mannans from different sources generally have different glycosidic bonds and different monosaccharide units. Therefore, the results of hydrolysis vary.

Structure variation can affect the activity of oligosaccharides. Garg *et al.* [10] noted that some activities are specifically exhibited by certain oligosaccharide structures, such as prebiotics, antibiotics, and vaccines.

Some prebiotics stimulate *Lactobacillus* sp. to a stronger degree than *Bifidobacteria* sp., while others stimulate both equally. This is also related to the structure of the oligosaccharide used [11].

Information about the type and abundance of each manno-oligosaccharide from the hydrolysis of mannan from konjac is another important aspect that needs elucidation, and mannanase produced by a microorganism needs to be characterized. This information would be beneficial as a reference for producing an oligosaccharide-based prebiotic. The aims of the present work are to determine the production time of mannanase from *S. lipmanii*, perform enzyme characterization, optimize the hydrolysis time, and characterize the hydrolysis product. Mannanase from *S. lipmanii* is expected to have a higher activity and be more suitable for hydrolyzing glucomannan from konjac.

Materials and Methods

Qualitative assay. Mannanolytic activity of *S. lipmanii* (isolate 724) was examined on solid medium contained 0.5 g konjac powder to which was added the following in 100 mL of distilled water: 0.075 g peptone, 0.05 g yeast extract, 1.5 g agar, 0.14 g $(NH_4)_2SO_4$, 0.2 g KH_2PO_4, 0.03 g $MgSO_4.7H_2O$, 0.03 g $CO(NH_2)_2$, 0.03 g $CaCl_2.2H_2O$, 0.0005 g $FeSO_4.7H_2O$, 0.00016 g $MnSO_4.7H_2O$, 0.00014 g $ZnSO_4.7H_2O$, and 0.0002 g $CoCl_2$.

The appearance of a clear zone was observed after 4-5 days of growth on solid medium by adding a solution of Congo red. The mannanolytic index (MI) was determined based on the ratio between the clear zone and colony diameters.

Enzyme assay in preculture and culture media. Liquid medium was used as the culture medium; its composition was the same as that of the solid medium without agar and with a konjac substrate. A single colony of *S. lipmanii* was cultivated in 20 mL preculture medium and then incubated for 24 h in a shaker at 30 °C and 150 rpm. Ten milliliters of the preculture medium was added to 90 mL of the culture medium for a total volume of 100 mL, and the solution was incubated again in a shaker for 5 days.

Sampling of the culture was performed every 24 h from the start day (D0) to the fifth day (D5). One milliliter sample was centrifuged at 4 °C at 13,750 g for 10 min, and then the enzyme-containing supernatant was stored at the same temperature.

Mannanase activity was examined by reacting 200 µL of 0.5% locust bean gum (LBG) with 200 µL of the mannanase sample followed by incubating for 15 min at room temperature. To this, 600 µL dinitrosalycylic acid (DNS) reagent was added, and the sample was heated at

100 °C for 15 min to stop the reaction. The control was prepared by reacting the substrate with DNS and then adding mannanase. After heating, the sample was allowed to cool down at room temperature, and the absorbance was measured at 540 nm [12]. A standard curve was made using mannose.

Enzyme production. Enzymes were produced by the same method as the preculture and culture and cultivated at the optimum time. For this study, a total volume of 600 mL culture was prepared. The culture was centrifuged at 4 °C at 13,750 g for 15 min. The crude enzyme supernatant was then stored at 4 °C until use.

Enzyme characterization. The enzyme was characterized by determining the optimum pH and temperature [13]. The optimum pH was determined by varying the pH from 4 to 10. The medium was adjusted to pH 3-6 with citrate buffer, pH 7-8 with phosphate buffer, and pH 9-10 with glycine-NaOH buffer.

Temperature optimization was carried out between 30 °C and 60 °C. The reaction between the enzyme and substrate (konjac 0.5% weight [w]/volume [v]) was carried out at a volume ratio of 1:1 for 15 min. Enzyme activity was determined by the DNS method.

Variation of hydrolysis time. Hydrolysis time of the substrate was varied from 0.5 to 5 h with a similar reaction as in the optimization process. Konjac 0.5% solution was used as the substrate. Reducing sugar and total sugar were used as the hydrolysis results and were determined by spectrophotometry.

Analysis of total sugar. The analysis of total sugar was performed using phenol [14] by reacting 200 µL of the sample solution with 200 µL 5% phenol solution and 600 µL concentrated H_2SO_4. The solution was then incubated for 15 min at room temperature and heated in a water bath at 40 °C for 30 min until its color changed. The reaction result was measured with a spectrophotometer at 490 nm.

Analysis of reducing sugar. Reducing sugar analysis was performed by the DNS method using a spectrophotometer at 540 nm.

Thin layer chromatography (TLC). An initial test to determine the type of oligosaccharides was performed by TLC. Mannose (**M1**) and mannotetrose (**M4**) were used as the standards. The tested samples were the following: enzyme (**E**), substrate (**S**), mixture of inactivated enzyme and substrate (**H0**), and the result after 2.5 h hydrolysis (**H2.5**). A mixture of diphenylamine, aniline, and phosphoric acid in acetone [15] was used as the reagent.

Analysis using High Performance Liquid Chromatography (HPLC). The oligosaccharides from enzymatic hydrolysis were qualitatively and quantitatively analyzed using HPLC. The Refractive Index Detector (RID) was used as the detector, and Zorbax-carbohydrate was used as the column. The mobile phase was acetonitrile and distilled water at a ratio of 60:40 v/v. The flow rate was 1 mL min^{-1}, and the temperature was 30 °C. The tested samples were **H0** and **H2.5**.

Results and Discussions

Mannanolytic activity by qualitative assay. Based on the qualitative assay, *S. lipmanii* on konjac medium produced a clear zone (Figure 1). After 4 days incubation, the diameter of the colony was 0.3 cm, while the diameter of the clear zone was 4 cm, giving an MI of 10.33.

Enzyme activity in preculture and culture media. Enzyme activity of each sample was measured every 24 h. Enzyme activity was high in the konjac liquid medium. Optimum activity of 8.488 U mL^{-1} was achieved on D_2 (Figure 2).

Enzyme characterization. The optimum pH of mannanase produced by *S. lipmanii* was pH 7 (Figure 3), whereas the optimum temperature of mannanase was 50 °C (Figure 4).

Figure 1. Mannanolytic Activity of *S. lipmanii* on Konjac Medium after 4 Days Incubation at 30 °C
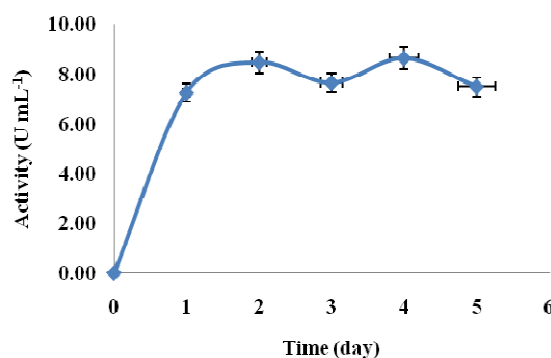

Figure 2. Enzyme Activity in Konjac Medium at 30 °C

Variation of hydrolysis time. Variation of hydrolysis time affected the reducing sugar content. Reducing sugar content increased with increasing hydrolysis time and reached an optimum at 2 h (Figure 5).

The ratio of total sugar to reducing sugar was used to prescribe the degree of polymerization (DP). The product of a 2-h hydrolysis process had a DP value of ~3 (Table 1). The DP value of oligosaccharides was between 2 and 9.
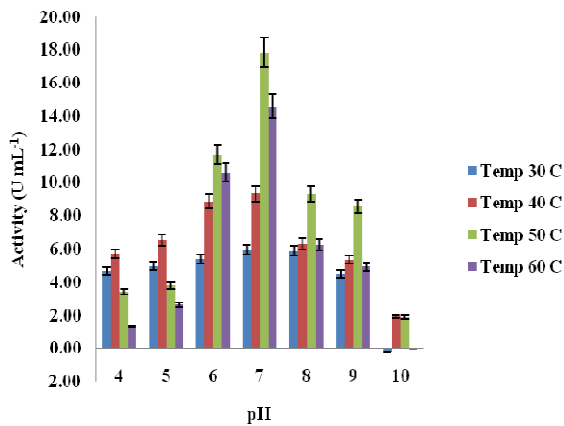
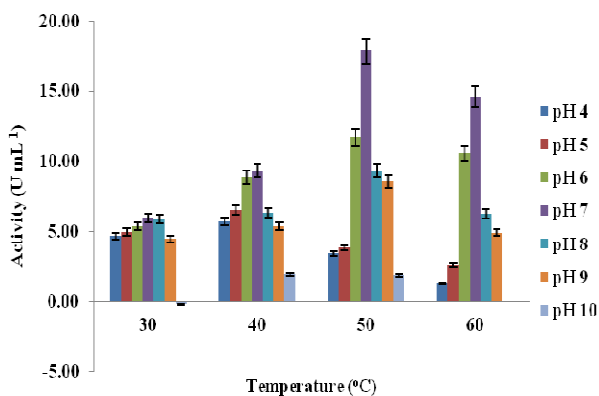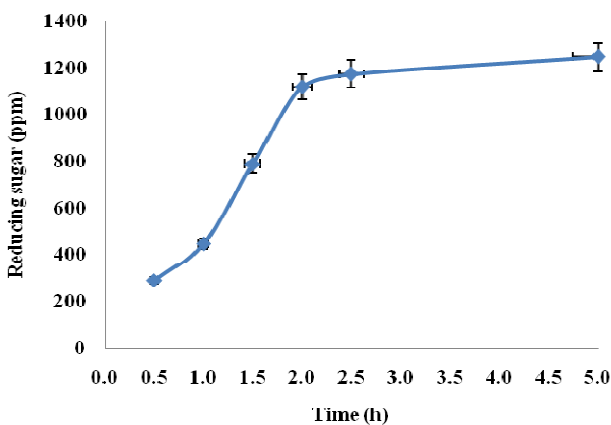**Thin layer chromatography.** Some new spots were generated from one of the samples from hydrolysis after 2.5 h (**H2.5**) (Figure 6). Some spots were assumed to be oligosaccharides because the retention factor (Rf) values were between 0.61 (mannose/**M1**) and 0.30 (manno-tetrose/**M4**) (Figure 6).

Figure 7 shows two peaks on the chromatogram of sample **H0**, which contained a mixture of inactivated enzyme and substrate. The first peak had a very small area (195 nRIU) with a retention time of 6.376 min, and the second peak had an area of 794 nRIU with a retention time of 7.379 min.

Figure 8 shows four peaks from konjac hydrolysis after 2.5 h. The retention time values of the peaks were 5.313, 5.849, 6.070, and 6.955 min.

Figure 3. Variation of Hydrolysis pH

Figure 4. Variation of Hydrolysis Temperature

Figure 5. Variation of Hydrolysis Time

Table 1. Reducing Sugar, Total Sugar and Degree of Polimerization (DP) with Varying Konjac Hydrolysis Time

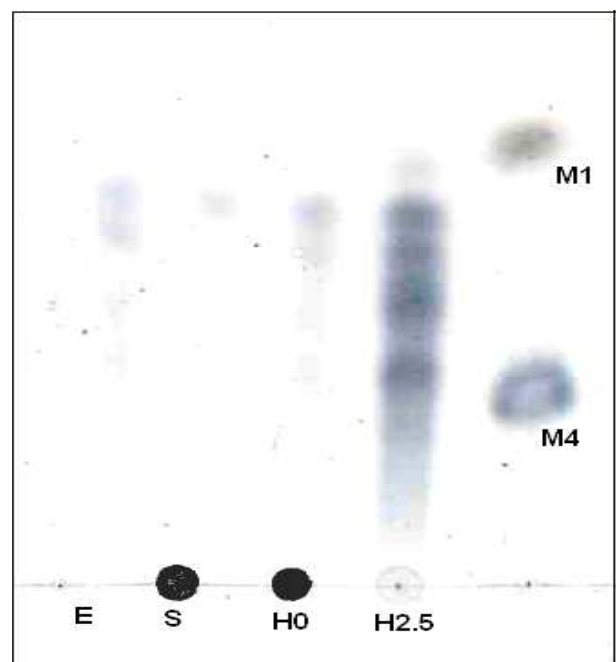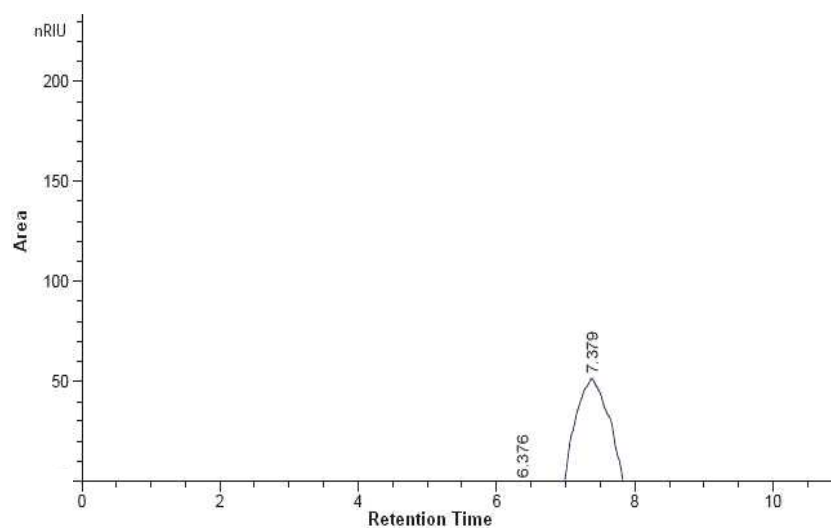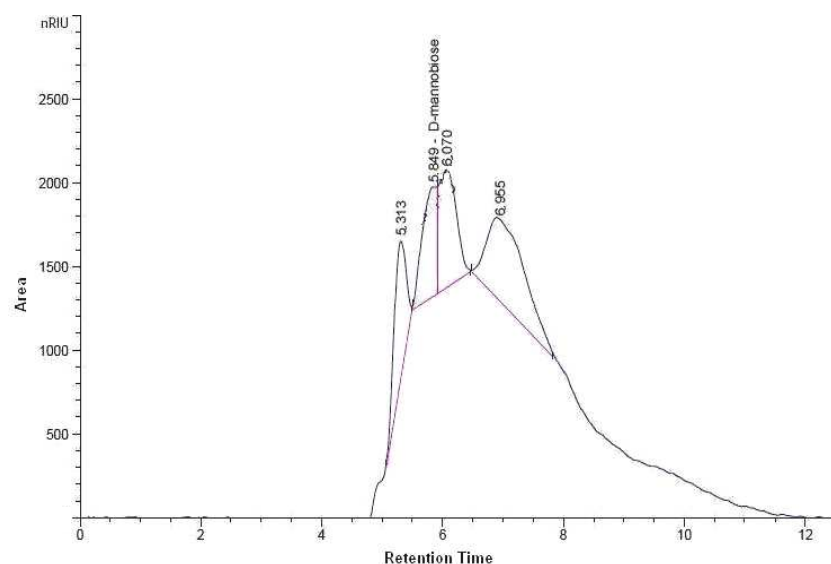| Hidrolysis Time (h)	Konjac		
	Reducing Sugar (ppm)	Total Sugar (ppm)	DP
0.5	290	3533	12.18
1.0	446	4040	9.06
1.5	790	3583	4.54
2.0	1118	3560	3.18
2.5	1174	3863	3.29
5.0	1246	3920	3.15

Figure 6.   TLC of Konjac (E: Enzyme; S: Substrate; M1:Mannose; M4: Mannotetrose)

Figure 7. Chromatogram of Inactivated Enzyme & Konjac Mixture (H0)

Figure 8. Chromatogram of Konjac after 2.5 h Hydrolysis (H2.5)

The Congo red solution used in the assay was a salt solution of a secondary diazo dye that has a stronger interaction with a polysaccharide than with an oligosaccharide or monosaccharide. A clear zone developed due to the weak interaction between compounds in the medium and Congo red (Figure 1). The clear zone around *S. lipmanii* indicated that mannose or manno-oligosaccharide was produced, indicating that *S. lipmanii* produced an enzyme that was able to hydrolyze mannan.

The MI value of this was 10.33. A higher MI value indicates higher mannanolytic activity. Phothichitto [16] reported that the average MI obtained from 19 isolated bacteria after 48 h incubation was 2.74 and the average obtained from four isolated fungi after 3-7 days incubation was 1.09.

The high value of enzyme activity suggests that konjac is a suitable substrate to induce enzyme production. Enzyme activity was not noticeably changed after incubation at D_2, possibly because the microorganism had entered the stationary phase where the presence of a nutrient source, such as mannan, was scarce. The activity began to drop after D_5, possibly because the micro-organism was in the death phase where the number of dying cells was higher than that of living cells [17].

The enzyme activity of 8.488 U mL^{-1} with konjac was much higher than that produced by *S. lipmanii* in palm

kernel cake (0.032 U mL^{-1}) [5]. Konjac was more suitable for enzyme production than palm kernel cake because konjac does not contain lignin, a compound that consists of phenolic groups and that was able to inhibit microorganism activity [17].

Based on the enzyme activity curve, the enzyme produced in the konjac liquid medium and harvested on D_2 had the optimum time of enzyme production by *S. lipmanii*.

The enzyme has a dissociation constant of the acid groups or basic groups, especially in the carboxyl terminal residues and amino acids. Changes in pH could affect the conformation of the enzyme, which would influence activity. Mannanase enzyme produced by *S. lipmanii* is likely to have the best conformation at pH 7, which is similar to the soil pH range where *S. lipmanii* was isolated.

Based on the temperature optimization results, the trend of enzyme activity increased from 30 °C to 40 °C. The temperature was optimized at 50 °C; lower enzyme activity was observed at 60 °C.

An increase in temperature might increase the reaction rate because as more energy is available for the molecules, there is a tendency to move. However, for an enzyme, which is classified as protein, a temperature increase can also decrease activity [18] because of enzyme conformational changes. Therefore, in addition to pH, temperature also determines enzyme activity.

The optimum time of hydrolysis was achieved at the second hour at which time the reducing sugar content from hydrolysis of konjac was 1118 ppm. After the second hour no significant increase was observed.

Reaction on phenol method to determine total sugar was analogous to the Molisch test. The addition of concentrated sulfuric acid induced the hydrolysis of polysaccharides to monosaccharides and then dehydration to form furfural. Furfural molecule binds with two molecules of phenol, and further reaction leads to the initial formation of more conjugated double bonds that caused an orange color [19].

The DP offers initial information on how long oligosaccharide chains were formed from the hydrolysis. A DP of 3-12 was recorded from the hydrolysis of mannan from konjac. Based on the DP, we concluded that oligosaccharides had formed completely. In this case, DP was an average value since the hydrolysis results were based on mixtures.

Spots on TLC (Figure 6) were assumed to be from hydrolysis (**H2.5**) since these spots were not observed in the controls (in the form of **E**, **S**, and **H0**). The variation of the spot products showed that the enzyme worked randomly within hydrolyzed mannan, including the endo-type enzyme.

Based on the HPLC chromatogram (Figure 7), the first peak had a retention time of 6.376 min, which lies between the retention time of mannobiose and mannotriose. The second peak in the chromatogram had a retention time of 7.379 min, which lies between the retention time of mannotriose and mannotetrose. These peaks could have been present before hydrolysis because the enzyme was produced in konjac medium.

In Figure 8, the first peak had a retention time of 5.313 min, which was located between the retention time of mannose and mannobiose. However, this peak is closer to the glucose retention time of 5.006 min, possibly because konjac contains glucomannan, which consists of mannose and glucose. The second peak was strongly predicted to be mannobiose at 756 ppm, and the third peak had a retention time between those of mannobiose and mannotriose.

The presence of glucose in the glucomannan chain allowed for the formation of a disaccharide from hydrolysis in addition to mannobiose and a trisaccharide other than mannotriose. Similarly, the fourth peak had a retention time between mannotriose and mannotetrose and was possibly a heterogeneous trisaccharide or tetrasaccharide.

The hydrolysis results, which were estimated as heterogeneous disaccharide, trisaccharide, and tetrasaccharide, had similar polarities and therefore could not be separated completely in the HPLC column. Condition optimization is required to separate each peak of the oligosaccharides and to make qualitative and quantitative determinations. The method of addition standard (known as spike) could also be performed to confirm compounds in the results.

Conclusions

Mannanase enzyme could be produced in konjac medium by *S. lipmanii* on the second day of culture. The optimum hydrolysis was achieved at pH 7 at a temperature of 50 °C, while the optimum time for hydrolysis of mannan from konjac was 2 h. Hydrolysis products were oligosaccharides with a retention time between those of mannose and mannotetrose.

Acknowledgment

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