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Sweet potato β-amylase immobilized on chitosan beads and its application in the semi-continuous production of maltose

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Abstract

The present study is concerned with the immobilization of β-amylase from sweet potato tuberous roots on chitosan beads for the purpose of maltose production. Immobilized sweet potato β-amylase on Chitopearl BCW 3505 beads exhibited an activity of 142 U/g carrier. The optimum temperature of the immobilized β-amylase increased by 20°C, and thermostability was improved by about 10°C compared to free β-amylase. The additional effects of two types of the immobilized pullulanases on maltose formation were also investigated. The addition of pullulanase from *Bacillus brevis* gave a higher yield of maltose compared to that from *Klebsiella pneumoniae*. A semi-continuous production of maltose from a highly concentrated substrate (Pine-Dex #1, potato starch hydrolyzate) solution (40%, w/v) with the immobilized sweet potato β-amylase and *Bacillus brevis* pullulanase could be obtained at pH 6.0 and 60°C for 14 days at a higher yield (70.9%) using a horizontal rotary column reactor.

Keywords: Chitosan beads; β-amylase; *Klebsiella pneumoniae*

1. Introduction

β-amylase, distributed in higher plants and microorganisms, can attack the α-1,4-linkages from the non-reducing ends of starch molecules, giving maltose units. β-amylase is used for production of high maltose syrup from starch in combination with pullulanase, which can hydrolyze the α-1,6-linkages in α-1,6-branched α-1,4-glucans. Maltose finds a wide range of applications in the food and pharmaceutical industries, since its properties are represented by mild sweetness, good thermal stability, low viscosity in solution, and lack of color formation.

To date, β-amylases from soybean, barley, and wheat have been used industrially. However, soybean β-amylase is relatively expensive, and barley and wheat β-amylases are lacking in thermostability. The sweet potato is thought to be a promising source of β-amylase since β-amylase is one of the major proteins in the tubers. Moreover, it has advantages as a crop in being resistant to unfavorable environments such as typhoons, drought, pests, and diseases. We have been studying intensively the properties and high utilization of sweet potato starch (Noda, Ohtani, Shiina & Nawa, 1992; Noda, Takahata, Sato, Hisamatsu & Yamada, 1995; Noda, Takahata, Sato, Ikoma & Mochida, 1997) and cell wall polysaccharides (Noda, Takahata, Nagata & Shibuya, 1994a; Noda, Takahata & Sato, 1994b). Additionally, fundamental properties of sweet potato β-amylase were studied also to some extent (Takahata, Noda & Nagata, 1994). This investigation focused on industrial applications of sweet potato β-amylase.

Industrial production of maltose is performed generally in a batch reaction, which is economically disadvantageous since the enzyme can be used only once. To allow enzyme reuse, enzyme immobilization has been used currently in industries. A wide variety of carriers to immobilize enzyme can be developed for this purpose. Among them, chitosan, a deacetylated derivative of chitin, appears to be a promising support material for use in the food industry because its non-toxic, cheap, easy to operate, and has a low rate of biodegradability. It was reported that chitosan was excellent for the immobilization of carbohydrate-degrading enzymes (Kimura, Yoshida, Oishi, Ogata & Nakakuki, 1989; Kuriki, Yanase, Takata, Takesada, Imanaka & Okada, 1993; Kuriki, Yanase, Takata & Okada, 1997; Muzzarelli, Barontini & Rocchetti, 1978; Shin, Park & Yang, 1998; Spagna, Andreani, Salatelli, Ramagnoli & Pifferi, 1998), including β-amylase (Yoshida, Oishi, Kimura, Ogata & Nakakuki, 1989). In addition, the immobilized enzyme on chitosan

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beads is likely to exhibit increased thermostability compared to the free enzyme (Kimura et al., 1989; Spagna et al., 1998). Higher temperature is desirable during the industrial enzyme reaction for prevention of microbial contamination.

The present study is concerned with the immobilization of β-amylase from sweet potato tuberous roots on chitosan beads for the effective production of maltose.

2. Materials and methods

2.1. Materials

Sweet potato (*Ipomoea batatas*) plants were grown during the summer months in an experiment field of the Sweet Potato Breeding Laboratory, Kyushu National Experiment Station, Miyakonojo, Miyazaki Prefecture, Japan. The cultivar used was Kyukei 90105-14, since it was known to accumulate a large amount of β-amylase in the tuberous roots compared with other cultivars. Tuberous roots were harvested, thoroughly washed, cut into small pieces, freeze-dried, and milled. The flour was stored at −4°C before use. For the extraction of β-amylase, 200 g of the flour was added to 400 ml of a 0.1 M acetate buffer (pH 6.0), and the mixture was held for 1 h at room temperature with stirring. After centrifugation (2000*g*, 10 min), the supernatant was used as the β-amylase preparation. The pullulanase preparation from *Klebsiella pneumoniae* was a gift from Amano Pharmaceutical Co., Ltd. The thermostable pullulanase preparation from *Bacillus brevis*, which was recently found, was kindly donated by Daiwa Kasei Co., Ltd. Porous chitosan beads (Chitopearl BCW series from Fuji Spinning Co., Ltd) were used for immobilization of the enzyme. Soluble starch, pullulan and Pine-Dex #1 (potato starch hydrolyzate, DE 8) are the products of Merck Co., Ltd, Wako Pure Chemical Industries Co., Ltd, and Matsutani Chemical Industry Co., Ltd, respectively.

2.2. Assay of free enzyme activity

The free β-amylase activity was assayed as follows. An enzyme solution (62.5 μl), a substrate solution (125 μl) of 1% (w/v) soluble starch and a 0.1 M acetate buffer (pH 6.0, 62.5 μl) were mixed and incubated for 10 min at 40°C. The reaction was terminated by the addition of a 3,5-dinitrosalicylic acid (DNS) solution (750 μl) (Imanaka & Kuriki, 1989), then heated in a boiling water bath for 5 min. After cooling, distilled water (3.0 ml) was added. The absorbance was measured at 540 nm with maltose as the standard. One unit of β-amylase activity corresponds to the amount that liberated 1.0 μmol of maltose per minute under the assay conditions. The free pullulanase activity was assayed as follows. An enzyme solution (62.5 μl), a substrate solution (125 μl) of 1.0% (w/v) pullulan, and a 0.1 M acetate buffer (pH 6.0, 62.5 μl) were mixed and incubated for 10 min at 40°C. The reducing sugar produced was analyzed by a DNS method as described for the β-amylase assay. One unit of pullulanase activity corresponds to the amount that liberated 1.0 μmol of maltose per minute under the assay conditions.

2.3. Optimum conditions of free β-amylase activity

The optimum pH of free β-amylase was determined as the relative activity after incubation for 10 min at 40°C. The reaction mixture consisted of 62.5 μl of an enzyme solution, 62.5 μl of a 0.1 M buffer of various pH values, and 125 μl of a 1.0% (w/v) soluble starch. To determine the pH stability, 0.1 ml of an enzyme solution was treated with 0.9 ml of a 0.1 M buffer of various pH (pH 4.0–11.0) values for 30 min at 40°C, and then the remaining β-amylase activity was analyzed. The optimum temperature of free β-amylase was assayed as the relative activity after treatment for 10 min in incubation at various temperatures (20–80°C). The reaction mixture consisted of 62.5 μl of enzyme solution, 62.5 μl of a 0.1 M acetate buffer (pH 6.0), and 125 μl of a 1.0% (w/v) soluble starch. The thermostability of free β-amylase was determined by treating the enzyme solution for 30 min in incubation at various temperatures (20–80°C), and then the residual β-amylase activity was analyzed at 40°C.

2.4. Immobilization of the enzymes

Chitopearl BCW series beads, porous chitosan beads that were cross-linked with an aliphatic or aromatic compound, were used as a carrier for enzyme immobilization. The carrier beads (diameter, 0.3 mm) were washed with water and a 0.1 M sodium acetate buffer (pH 6.0). The enzymes used were immobilized on the beads by simple adsorption. In the case of β-amylase, a sample of 100 mg (wet basis) of four kinds of chitosan beads (Chitopearl BCW 2505, -2605, -3005, and -3505) and 3.0 ml of a solution containing 200 U of β-amylase in the same buffer were mixed. The mixture was shaken for 1 h at room temperature. After centrifugation (2000*g*, 10 min), the β-amylase activity of the supernatant was assayed under standard conditions. Two types of commercial pullulanases were used for the immobilization test. One originated from *Klebsiella pneumoniae* and the other from *Bacillus brevis*. Two hundred units of each of pullulanase was applied per 1 g (wet basis) of chitosan beads (Chitopearl BCW 3505) and shaken for 1 h at room temperature. After centrifugation (2000*g*, 10 min), the pullulanase activity of the supernatant was assayed under standard conditions. The apparent activity of the immobilized enzyme was expressed as follows: apparent activity of the immobilized enzyme (U/g) = (enzyme activity applied) − (enzyme activity in the supernatant). The ratio of the immobilization of the enzyme was calculated as follows: Ratio of immobilization of enzyme (%) = [(apparent activity)/(enzyme activity applied)] 100. The immobilized enzyme obtained in the above procedure was washed thoroughly with a 0.1 M sodium acetate buffer (pH 6.0). Then, the activity of the immobilized enzyme was determined as

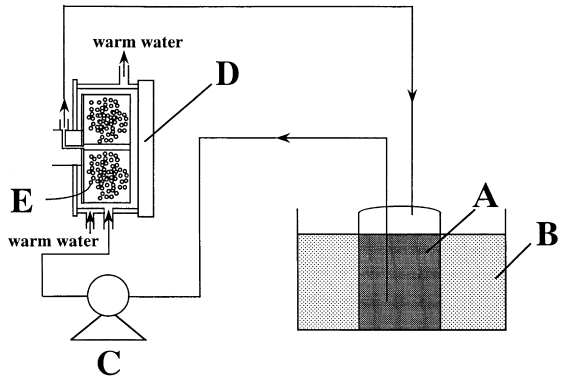

Fig. 1. Schematic diagram of a horizontal rotary column reactor: (A) substrate solution; (B) water bath; (C) peristaltic pump; (D) horizontal rotary column; and (E) immobilized enzyme.

described below. This activity is regarded as the exhibited activity of the immobilized enzyme.

2.5. Assay of the immobilized enzymes

The activities of the immobilized β-amylase and the immobilized pullulanase were measured as follows. For immobilized β-amylase, 10 mg (wet basis) of the immobilized enzyme was suspended in 0.5 ml of a 10 mM acetate buffer (pH 6.0), and then 5.0 ml of a substrate solution of 10% (w/v) soluble starch was added to the mixture. After incubation at 40°C for 20 min, the amount of reducing sugar in the supernatant was measured by the DNS method. This activity was defined as the exhibited activity of the immobilized β-amylase. The activity of the immobilized pullulanase was determined in the same way as the immobilized β-amylase using 10% (w/v) pullulan instead of 10% (w/v) soluble starch.

2.6. Optimum conditions of immobilized β-amylase

The optimum pH was assayed as a relative activity after incubation for 20 min at 40°C. The reaction mixture consisted of 10 mg (wet basis) of the immobilized enzyme, 0.5 ml of a 10 mM of buffer of various pH values, and 5.0 ml of a substrate solution of 10% (w/v) soluble starch. To determine the pH stability, 20 mg (wet basis) of immobilized β-amylase was added to 2.0 ml of a 0.1 M buffer of various pH values. The mixture was allowed to stand for 30 min at 40°C. After centrifugation (2000*g*, 10 min), the supernatant was discarded. Ten milligrams (wet basis) of the treated immobilized enzyme was added to 0.5 ml of a 10 mM buffer (pH 6.0) and 5.0 ml of a substrate solution of 10% (w/v) soluble starch and was treated for 20 min at 40°C. The optimum temperature was assayed by incubating the immobilized enzyme with 0.5 ml of a 10 mM acetate buffer (pH 6.0) and 5 ml of a substrate solution of 10% (w/v) soluble starch at various temperatures for 20 min. To determine the thermostability, 10 mg (wet basis) of immobilized β-amylase was added to 0.5 ml of an acetate buffer (pH 6.0). The mixture was allowed to stand for 30 min in an incubator at various temperatures (20–80°C). Then, 5.0 ml of a substrate solution of a 10% (w/v) soluble starch was added and the residual activity was assayed after treatment at 40°C for 20 min.

2.7. Production of maltose using a horizontal rotary column reactor

A horizontal rotary column reactor, developed by Honda, Hashiba, Ahiko and Takahashi (1991), was used for semi-continuous production of maltose with the immobilized sweet potato β-amylase and *Bacillus brevis* pullulanase. The schematic diagram of the reactor is shown in Fig. 1. In a 1 l vessel, 400 g of Pine-Dex #1 (40%, w/v) suspended in a 25 mM acetate buffer (pH 6.0) to a final volume of 1 l was supplied. After 11.3 g (wet basis) of immobilized sweet potato β-amylase (1600 U) and 15.2 g (wet basis) of immobilized *Bacillus brevis* pullulanase (1300 U) were placed in the column chamber, the substrate solution was circulated by operating a peristaltic pump to contact these immobilized enzymes. The column temperature was maintained at 60°C using a circulator, and the vessel was at the same temperature using a water bath. The flow rate was kept constant (6.4 l/h) by using a peristaltic pump. The enzyme reaction was carried out at a rotational speed of 60 rpm. When the maltose content reached about 30%, which was close to the content at maximum according to our preliminary experiment, the substrate solution was withdrawn by operating a peristaltic pump in the opposite direction. The next enzyme reaction was started by supplying a fresh substrate solution in the vessel.

2.8. Carbohydrate analysis

The linear maltosaccharides released after enzyme treatment were analyzed by high performance anion exchange chromatography (HPAEC) using the Dionex BioLC system equipped with a pulsed amperometric detector (PAD). The column used was CarboPac PA-1 (4×250 mm) with CarboPac PA-1 guard column. The chromatogram was analyzed with a Hitachi D-2500 chromato-integrator. Prior to analysis, an internal standard (6-deoxyglucose) solution was added to each hydrolyzate. The samples were then filtered through a 0.22 μm membrane filter. Samples were eluted at 1.0 ml/min with a linear gradient of 150 mM NaOH (100–70%) and 150 mM NaOH containing 500 mM sodium acetate (0–30%) for 30 min. In this system, glucose, maltose, isomaltose, and maltotriose were well separated. The yield of maltose was defined as a weight percentage of maltose produced based on the weight of the total saccharides in the sample.

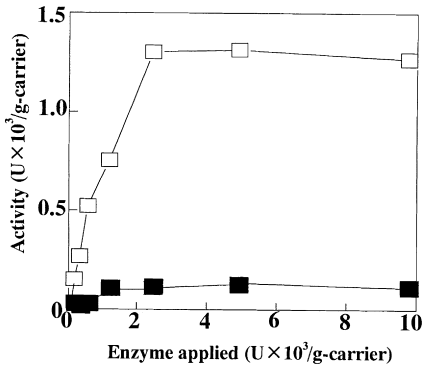

Fig. 2. Relationship between amount of β-amylase applied and apparent (□) and exhibited (■)activities of immobilized β-amylase.

3. Results and discussion

3.1. Immobilization of sweet potato β-amylase

To establish the simplified procedure for the preparation of the immobilized β-amylase from an agricultural product, we used intact sweet potato tubers, not a commercial β-amylase preparation, in this investigation. It has been reported that α-amylase may reduce the yield of maltose during starch hydrolysis by β-amylase (Thacker, Ramamurthy & Kothari, 1992). Since sweet potato tuberous roots accumulate α-amylase in addition to a large amount of β-amylase (Hagenimana, Vezina & Simard, 1992; Takahata, Noda & Sato, 1995), we were afraid of α-amylase contamination in the sweet potato β-amylase preparation. For this reason, we used the cultivar, Kyukei 90105-14, which contains a very small amount of α-amylase. Takahata et al. (1995) reported that α-amylase activity tended to increase during storage. Therefore, soon after harvesting the sweet potato, we prepared the flour. After the extraction of β-amylase from the sweet potato tubers, a β-amylase preparation having 163 U/ml was obtained and used for the immobilization test. As the β-amylase preparation showed very little α-amylase activity (data not shown), its further purification was not necessary.

Among the four kinds of chitosan beads, BCW 3505 showed the highest ratio of immobilization (58.9%). Furthermore, the immobilized β-amylase on BCW 3505 beads had the highest exhibited activity (data not shown). Accordingly, we selected BCW 3505 beads for the immobilization of β-amylase. BCW 3505 beads have aromatic alkyl groups for hydrophobic bonding with proteins as well as amine group for ionic bonding with them. The effect of the shaking time during immobilization on the ratio of immobilization was also evaluated. The ratio of immobilization of β-amylase reached a constant value after more than 60 min (data not shown). Fig. 2 shows the relationship between the amount of β-amylase applied and the apparent and exhibited activities of the enzyme immobilized on BCW 3505 beads. As the amount of β-amylase applied per 1 g carrier increased, the exhibited activity of the immobilized enzyme increased and reached an equilibrium (about 120 U/g carrier) when more than 2000 U of β-amylase per 1 g of carrier was added. On the other hand, the apparent activity increased linearly with an increase in the amount of β-amylase applied, and the activity reached an equilibrium (about 1300 U/g carrier) when more than 2000 U of β-amylase per 1 g carrier was added. The apparent activity of the immobilized β-amylase was about ten times as large as the exhibited activity when more than 2000 U of β-amylase per 1 g carrier was used. It is suspected that the reason for this is that the sweet potato β-amylase coupled with the macroporous structure of the carrier is not thoroughly active because the starch does not permeate the inner part of the beads, as suggested by Yoshida, Kimura, Ogata and Nakakuki (1988). From the results obtained here, we set up standard conditions for the immobilization of sweet potato β-amylase on Chitopearl BCW 3505 beads as follows: (i) 2000 U of β-amylase were applied per 1 g of carrier; (ii) the shaking time during the immobilization treatment was 60 min at room temperature. Under standard conditions, we obtained 142 U of exhibited activity per 1 g of carrier of the immobilized β-amylase on a larger scale. Yoshida et al. (1989) obtained 134 U of exhibited activity per 1 g of carrier of the immobilized β-amylase using a commercial soybean β-amylase preparation and Chitopearl BCW 3505 beads. Thus, we established a way to immobilize β-amylase with a fairly good activity using intact sweet potato tubers and chitosan beads.

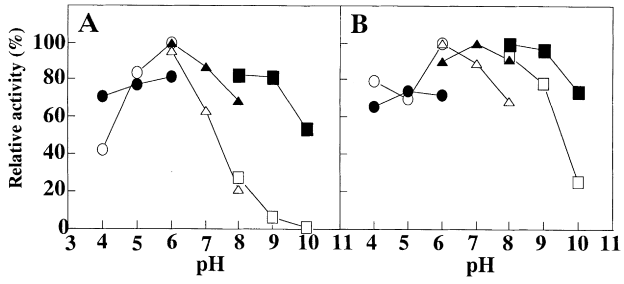

Fig. 3. Effect of pH on activity and stability of free (open symbol) and immobilized (closed symbol) β-amylase. (A) Effect of pH on enzyme activity. The activity was measured in a sodium acetate buffer (○, ●) a Tris-HCl buffer (△, ▲), or a borate buffer (□, ■) for 30 min at 40°C. The highest activity is regarded as 100%. (B) Effect of pH on stability of enzyme. The enzyme was treated in a sodium acetate buffer (○, ●), a Tris-HCl buffer (△, ▲), or a borate buffer (□, ■) for 30 min at 40°C. The remaining activity was assayed.

3.2. Properties of the immobilized sweet potato β-amylase

We attempted to examine the properties of the immobilized β-amylase and to compare them with those of the free enzyme. The pH profile on the activity and stability of the immobilized β-amylase compared to the free enzyme is presented in Fig. 3. The optimum pH of the immobilized β-amylase was similar to that of the free enzyme. The immobilized β-amylase activity was higher than that of

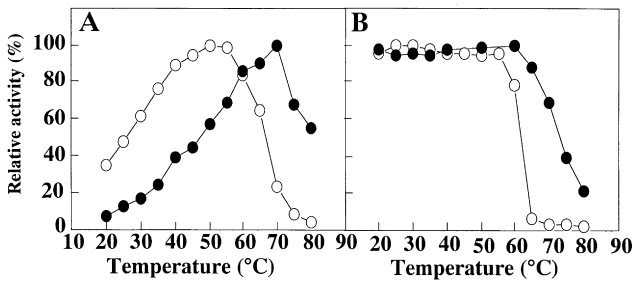

Fig. 4. Effect of temperature on activity and stability of free (○) and immobilized (●) β-amylase. (A) Effect of temperature on enzyme activity. The activity was measured at various temperatures (20–80°C). The highest activity is regarded as 100%. (B) Thermal stability of the enzyme. The enzyme was treated at various temperatures (20–80°C) for 30 min. The remaining activity was assayed at 40°C.

the free β-amylase on the alkaline side (pH 7.0–10.0). Both activities of the free and immobilized β-amylase were stable over the pH range of 4.0–9.0. Fig. 4 shows the temperature effects on the activity and stability of free and immobilized β-amylase. The optimum temperature of the immobilized β-amylase was higher (70°C) than that of the free enzyme (50°C). Thermostability of β-amylase was improved by immobilization and increased by about 10°C compared to the free enzyme. Immobilized β-amylase was found to be quite stable up to 60°C without a significant loss of activity. The reaction temperature for the digestion test was set at 60°C to prevent microbial contamination. Unlike the results obtained here, soybean β-amylase was reported to show little increase in thermostability through the immobilization of chitosan beads (Yoshida et al., 1989).

3.3. Additional effects of pullulanase on maltose production

In this study, two types of pullulanase preparations were immobilized on Chitopearl BCW 3505 beads, which were reported to be the best carrier for the immobilization of pullulanase (Kimura et al., 1989). The ratio of immobilization of pullulanase from *Klebsiella pneumoniae* and that from *Bacillus brevis* were 93.9 and 86.1%, respectively, when 200 U of pullulanase were added per 1 g carrier. The exhibited activities were 58.1 and 85.5 U/g carrier for pullulanase from *Klebsiella pneumoniae* and *Bacillus brevis*, respectively.

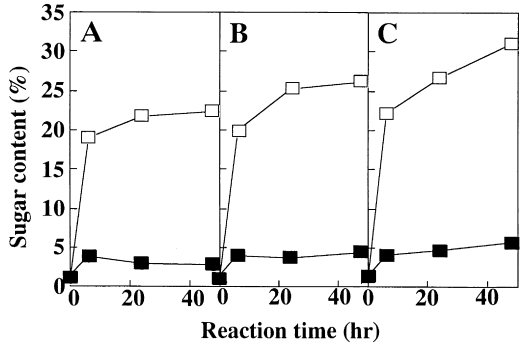

Fig. 5. Effect of two types of the immobilized pullulanases on maltose formation. Conditions: substrate (Pine-Dex #1) concentration, 40% (w/v); immobilized sweet potato β-amylase activity, 16 U; temperature, 60°C; pH, 6.0; total volume, 1 l. Thirteen units of immobilized pullulanase was added from the onset of reaction. Pullulanase: (A) control without pullulanase; (B) *Klebsiella pneumoniae* pullulanase; and (C) *Bacillus brevis* pullulanase. □, maltose; ■, maltotriose.

We used Pine-Dex #1, potato starch hydrolyzate, as a substrate for maltose production. Pine-Dex #1 contained small amounts of maltose (2.72%) and maltotriose (1.95%). In our preliminary test, the influence of a high concentration of substrate solution on the rate of maltose formation was examined by batch digestion with the immobilized β-amylase. The maltose production rate increased linearly with an increase in the initial substrate concentration from 10 to 40% (w/v). Consequently, the initial substrate concentration for the following digestion test was set at 40% (w/v) to obtain a higher production rate of maltose. Batch digestions were performed to evaluate the additional effects of two types of immobilized pullulanases on the increase in maltose formation by the immobilized β-amylase. Samples were removed for measurement of sugar composition at intervals of up to 48 h, and the results are presented in Fig. 5. Sugar composition of products obtained from a batch reaction with 40% (w/v) of a substrate (Pine-Dex #1) solution was analyzed by HPAEC. In the control (without pullulanase), the maltose content reached 22.3% at 48 h. With immobilized *Klebsiella pneumoniae* pullulanase, the maltose content was increased by 3.8% at 48 h and with *Bacillus brevis* pullulanase, the increase was by 8.8% compared to the control. It was reported that the optimum temperature of the immobilized *Klebsiella pneumoniae* pullulanase was 55°C; about 50% of its original activity was present at 55°C, and only about 15% at 60°C (Kimura et al., 1989). Therefore, thermo-inactivation of immobilized *Klebsiella pneumoniae* pullulanase was assumed to occur during saccharification at 60°C. Thus, the addition of the immobilized *Bacillus brevis* pullulanase proved to be more effective for the production of maltose with the immobilized sweet potato β-amylase. In all cases, HPAEC analysis showed that maltotriose was present at 12–18% of the maltose level and that glucose was not present.

3.4. Production of maltose using a horizontal rotary column reactor

Semi-continuous production procedure, that is, repeated batch production of maltose from 40% (w/v) of a substrate solution was performed using the immobilized sweet potato β-amylase and *Bacillus brevis* pullulanase. A horizontal rotary column reactor was operated at 60°C for 14 days, and the operation results are shown in Fig. 6. After the maltose content reached about 30% at 48 h, the substrate solution was withdrawn at 48 h periods, analyzed for sugar composition by HPAEC, and then a fresh substrate solution was supplied. As can be seen in Fig. 6, the maltose content was maintained at about 30%, which corresponds to 71.4%

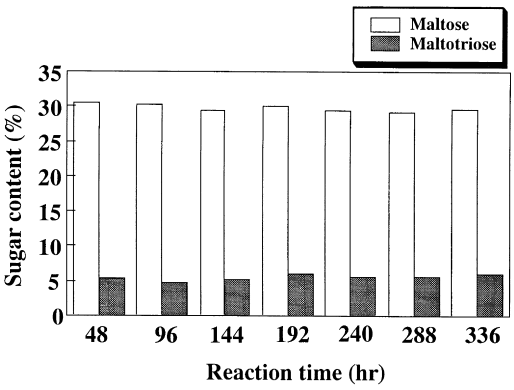

Fig. 6. Semi-continuous production of maltose with a horizontal rotary column reactor. A substrate solution was emptied and then refilled at every 48 h of reaction time. Conditions: substrate (Pine-Dex #1) concentration, 40% (w/v); immobilized sweet potato β-amylase activity, 1600 U; immobilized *Bacillus brevis* pullulanase activity, 1300 U; temperature, 60°C; pH, 6.0; total volume, 1 l.

of the maltose yield, even with the increased number of reaction cycles. Therefore, we assumed that there was little leakage of the enzyme over the period of the continuous operation. The yield of maltose was 70.9% (average of all seven runs) during the 14 day operation.

Yoshida et al. (1989) reported on the continuous production of maltose using the immobilized soybean β-amylase and *Klebsiella pneumoniae* pullulanase. They observed that a high reaction temperature (60°C) led to an obvious inhibition of maltose formation for a longer saccharification period (150 h), presumably due to the low thermostability of the immobilized *Klebsiella pneumoniae* pullulanase. As was found in the batch test, maltotriose was produced at 16–20% of the maltose level in all runs. Maltotriose is inevitably produced in starch saccharification by a combination of β-amylase and pullulanase (Shaw & Sheu, 1992). Further work is needed to reduce the proportion of maltotriose, since the maltotriose in maltose syrups often limits their use particularly in the pharmaceutical industry. Although microbial contamination is generally a problem in the utilization of an immobilized enzyme, no contamination by microorganisms was observed in this study, presumably because of the high reaction temperature (60°C) and the high concentration of substrate (40%).

β-amylase prepared from sweet potato tuberous roots was immobilized successfully on chitosan beads and was used for an effective production of maltose. With this procedure, the activity of the immobilized β-amylase was found to be fairly good (142 U/g carrier). More importantly, the immobilized β-amylase showed higher thermostability compared to the free one. The addition of the immobilized pullulanase from *Bacillus brevis* had larger effects on the increase in maltose formation by the immobilized β-amylase compared to that from *Klebsiella pneumoniae*. A horizontal rotary column reactor was run at 60°C of the reaction temperature with the immobilized sweet potato β-amylase and *Bacillus brevis* pullulanase. The yield of maltose from 40% (w/v) of the substrate solution was 70.9% during the 14 day operation.

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