Journal of Biochemistry and Molecular Biology, Vol. 35, No. 6, November 2002, pp. 568-575

JOURNAL OF Biochemistry and Molecular Biology

New Action Pattern of a Maltose-forming α-Amylase from *Streptomyces* sp. and its Possible Application in Bakery

Youssef Ben Ammar*, Takayoshi Matsubara, Kazuo Ito, Masaru Iizuka, Tipaporn Limpaseni†, Piamsook Pongsawasdi† and Noshi Minamiura

Laboratory of Enzyme Chemistry, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558, Japan

†Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

Received 26 July 2002, Accepted 20 August 2002

An α-amylase (EC 3.2.1.1) was purified that catalyses the production of a high level of maltose from starch without the attendant production of glucose. The enzyme was produced extracellularly by thermophilic *Streptomyces* sp. that was isolated from Thailand's soil. Purification was achieved by alcohol precipitation, DEAE-Cellulose, and Gel filtration chromatographies. The purified enzyme exhibited maximum activity at pH 6-7 and 60°C. It had a relative molecular mass of 45 kDa, as determined by SDS-PAGE. The hydrolysis products from starch had α-anomeric forms, as determined by ^{1}H-NMR. This maltose-forming α-amylase completely hydrolyzed the soluble starch to produce a high level of maltose, representing up to 90%. It hydrolyzed maltotetrose and maltotriose to primarily produce maltose (82% and 62%, respectively) without the attendant production of glucose. The high maltose level as a final end-product from starch and maltooligosaccharides, and the unique action pattern of this enzyme, indicate an unusual maltose-forming system. After the addition of the enzyme in the bread-baking process, the bread's volume increased and kept its softness longer than when the bread had no enzyme.

Keywords: Action pattern, α-Amylase, Bakery, Maltogenic mechanism

Introduction

There is considerable commercial interest for Alpha amylases that are capable of catalyzing the production of high yields of specific maltooligosaccharides on the degradation of starch (Collins *et al*., 1993). Maltose is one of these useful maltooligosaccharides. It is desired for its wide industrial application in the food, pharmaceutical, biomedical, and fine-chemicals industries (Fogarty *et al*., 1983).

Maltose-forming α-amylases have been isolated from both bacterial and fungal sources (Collins *et al*., 1993). Maltose is produced by α-amylases of thermophilic actinomycetes, including *Streptomyces thermoviolaceus* (Goldberg and Edwards, 1990), *Thermoactinomyces* sp. no.15 (Obi and Odibo, 1984), and *Thermomonospora curvata* (Collins *et al*., 1993). However, starch degradation by almost all thermostable amylases involves the synchronous production of appreciable levels of glucose (Doyle *et al*., 1989).

The α-amylase that is presented in this paper was secreted by *Streptomces* sp.; however, it could be considered an interesting enzyme in regard to its high level of maltose that is produced, and the special action pattern that is different from other α-amylases. The production of a very small yield of glucose in the final end-product may be explained by the participation of reactions other than simple hydrolysis. This enzyme presented promising results for use in the bread-baking process.

Materials and Methods

Materials Soluble starch and maltooligosaccharides that were used as substrates in this work were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan), Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Source of microorganism Various soil samples were collected from Thailand, and different microorganisms were screened from these samples for their amylolytic activities. One of them, which was identified as *Streptomyces* sp., was retained and used for the production of the α-amylase that is presented in this paper.

*To whom correspondence should be addressed.
Tel: 81-6-6605-2585; Fax: 81-6-6605-2522
E-mail: youssef@sci.osaka-cu.ac.jp

Screening medium The medium that was used for screening of thermophilic bacteria that produces α-amylase had the following composition (g/l): soluble starch, 10.0; polypepton, 5.0; KH_2PO_4, 1.0; $MgSO_4;7H_2O$, 0.5; and agar, 15. The initial pH was 7.0. All of the media were sterilized by autoclaving at 121°C for 20 min. The screening was performed at 55°C for 48 h.

Enzyme production The medium that was used for the enzyme production had the following composition (g/l): meat extract, 10.0; polypepton, 10.0; albumin, 4.0; NaCl, 5.0; and soluble starch, 50.0. The meat extract and polypepton were dissolved in 500 ml tap water, then the pH was adjusted to 5.0 with HCl. Next, albumin was added and the mixture was boiled for 30 min. The medium was cooled down, NaCl was added, and the volume was adjusted to 500 ml. Separately, the soluble starch was dissolved in 500 ml of tap water, then mixed with other components. Initial pH was 6.5. The pre-culture and main culture were incubated at 55°C, and shaken overnight. The cells were removed by centrifugation at 7000 rpm for 20 min. The resulting cell-free supernatant was used as the starting material for the enzyme purification. The α-amylase was excreted into the culture medium.

Enzyme purification All of the operations for enzyme purification were performed at 4°C. Centrifugation was conducted at 7000 rpm for 20 min. The crude enzyme solution was first concentrated by ethanol precipitation (2 × v), followed by dialysis against distilled water at 4°C overnight, then applied on a DEAE-cellulose column (3.5 × 15 cm) that was pre-equilibrated with a 0.02 M sodium acetate buffer pH 6.2. The adsorbed proteins were eluted in salt gradient (with 800 ml of 0 to 1 M NaCl). The fractions that showed α-amylase activity were pooled and dialyzed overnight against distilled water and lyophilized, then subjected to a gel filtration column of Bio-Gel P-100 (2.5 × 55 cm). The fractions that showed activity were combined, then applied again to a DEAE-cellulose column (3.5 × 15 cm) that had been equilibrated with a 0.02 M sodium acetate buffer pH 6.2. The elution was performed in two steps: the first step used 500 ml of 0 to 0.5 M sodium chloride gradient, the second step was carried out using 200 ml of 1 M sodium chloride solution. The fractions that contained enzyme activity were combined, dialyzed, and lyophilized. Two other successive gel filtrations on Bio-Gel P-100 were finally applied in order to obtain the pure enzyme preparation.

Electrophoresis and determination of M_r The homogeneity of the purified α-amykase was demonstrated using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Weber and Obsorn, 1975). The molecular mass of the purified enzyme was estimated using both SDS-PAGE and gel filtration on a column of Bio-Gel A1.5 (3 × 70 cm). The standard proteins that were used for the SDS-PAGE were a protein mixture from a Pharmacia low molecular-weight calibration kit (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa).

Enzyme assay Alpha amylase activity was assayed by incubating 0.5 ml enzyme solution with 0.5 ml of 1% (w/v) soluble starch in Britton and Robinsonís Universal buffer (Campbell, 1955), pH 6.0, at 50°C for 10 min. The reaction was stopped, and the reducing sugars were determined by the method of Somogyi-Nelson (Somogyi 1952). One unit of α-amylase was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute under standard conditions.

To determine K_m values, 0.5 ml of the enzyme solution was reacted with 0.5 ml of each substrate at different concentrations. The activity was then measured. The K_m values were calculated directly from the curves that represented 1/*v* versus 1/S, where *v* is the enzyme velocity and S is the substrate concentration.

Optimum pH and pH stability To determine the optimum pH of the purified enzyme, 0.5 ml of 1% soluble starch that was dissolved in Britton and Robinson's Universal buffer, pH 3.0-9.0, and 0.5 ml of the enzyme solution (0.27 U) were mixed. Enzyme activity was assayed by incubating the mixture at 50°C for 10 min. For measuring pH stability, a reaction mixture that contained 0.1 ml of the enzyme solution (27 U) and 0.9 ml of Britton and Robinsonís Universal buffer, pH 3.0-9.0, were kept at 4°C for 24 h. The remaining activities were assayed at 50°C for 10 min after the mixture was diluted with the same buffer pH 6.0.

Optimum temperature and thermal stability To determine the optimum temperature of the purified enzyme, 0.5 ml of 1% soluble starch that was dissolved in Britton and Robinson's Universal buffer, pH 6.0, and 0.5 ml of the enzyme solution (0.27 U) were reacted together. The optimum temperature was measured at a range from 30°C to 75°C for a 10-min reaction time. To determine themostability, 1.0 ml of the enzyme solution (0.27 U) was kept at various temperatures for 15 min, cooled down at room temperature, then the remaining activity was assayed at 50°C for 10 min.

Protein measurement The protein concentration was measured spectrophotometrically on a Hitachi spectrophotometer Model U-1100 by assuming that the absorbance of 1% enzyme solution was 10.0.

High-performance liquid chromatography (HPLC) A sugar analysis was done by HPLC using Shimpak SCR 101 N (0.79 × 30 cm) in distilled water as the mobile phase at a flow rate of 1.0 ml/min at 60°C. The sugars were monitored with a Knaur differential refractometer.

Thin layer chromatography (TLC) TLC was done using silica gel (Kiesel Gel 60, Merck). It was developed at ambient temperature for 4 h with the solvent system of 1-propanol-ethylacetate-water (7 : 1 : 2). Spots were visualized by dipping in a solution of ethanol-sulfuric acid (9 : 1), drying, and heating at 110°C for 10 min.

Nuclear magnetic resonance Identification of the anomeric forms of the products that were released by the enzyme from soluble starch was done by ^{1}H-NMR with a Varian-UNITY plus 500 NMR spectrometer that operated at 500 MHz in D_2O at ambient temperature. Chemical shifts were measured with sodium-4, 4-dimethyl-4-sila-pentane sulfonite (DSS) as the internal standard.

Dough preparation The bread-dough recipe included 2000 g of

Table 1. Purification steps of the maltose-forming α-amylase

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme*	38000	700	0.018	100	1
Alcohol precipitation	1200	700	0.58	100	32.2
1st DEAE-Cellulose	920	780	0.85	111	47.2
1st P-100	364	560	1.54	80	85.5
2nd DEAE-Cellulose	240	570	2.37	81	131.7
2rd P-100	40	500	12.5	71.4	694.5
3th P-100	27	412	15.2	58.8	844.5

*: Two liters of culture medium were used to generate the crude enzyme.

wheat flower, 120 g of sugar, 40 g of salt, 80 g of shortening, 60 g of compressed yeast, 0.06 g of ascorbic acid, and 1360 g of water. When α-amylase was added, then 5000 U of the enzyme was used. All of the ingredients were mixed in a mixing bowl for 20 min, left at 25°C for 20 min, then the dough was divided into sections of 450 g each. Fermentation was conducted at 38°C, 85% RH for 1 h. Finally, the loafs were baked at 210°C for 32 min.

Specific volume and softness of bread The bread loaf volume was measured 30 min after baking with the rape-seed-displacement method. The ratio volume/mass (specific volume) was then calculated.

To measure the softness of the bread during the time frame, crumbs of 2 cm thickness were prepared and kept in polyvinyl bags for 1, 3, and 7 d. The resistance power was then measured. The crumb was considered to be softer when the resistance values were small. All of the measures were performed at least three times.

Results and Discussions

Purification of the maltose-forming α-amylase and determination of the molecular mass The purification of the maltose-forming α-amylase was achieved by alcohol precipitation, and alternation between DEAE-Cellulose and Bio-Gel P-100 gel chromatographies, as described previously in the Materials and Methods Section. The enzyme was purified to an electrophoretically-pure state with a final yield of 58.8% and purification fold of 844.5. The results showing the purification steps are illustrated in Table 1.

The molecular mass (M_r) of the purified maltose-forming enzyme was 45 kDa and 49 kDa, as determined respectively by SDS-PAGE (Fig. 1) and gel filtration chromatography.

pH and temperature characteristics of the enzyme The enzyme activity was maximal at pH, between 6.0 and 7.0. However, the enzyme preparation retained more than 85% of its activity between pH 5.5 and 8.0. Maximal stability was observed between pH 5.0 and 7.0, while the remaining activity was more than 60% for a wide pH range, between 4.0 and 9.0 (Fig. 2A).

This maltose-forming enzyme showed maximum activity at 60°C, and the thermal stability was up to 40°C. However, the remaining activity was about 93% and 82%, respectively at 45 and 50°C (Fig. 2B).

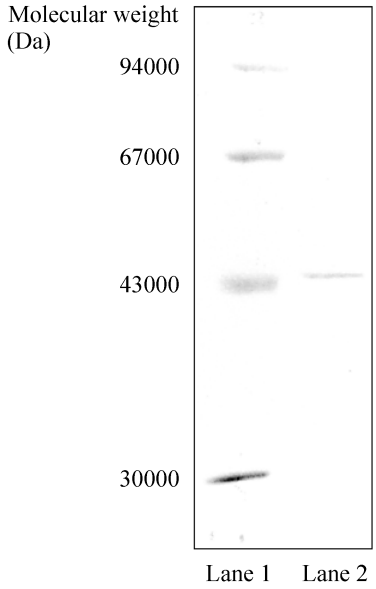

Fig. 1. SDS-PAGE of the purified maltose-forming α-amylase. Lane 1: molecular weight markers. Lane 2: the purified maltose-forming α-amylase obtained from the last Bio-Gel P-100. Twenty μg of the enzyme were applied on 10% polyacrylamide gel in a Tris-HCl buffer (pH 8.8). Electrophoresis was carried out at 20 mA for 130 min.

Determination of the anomeric forms of the enzyme products from soluble starch To determine the anomeric forms of the products, 13 units of the purified enzyme were added to 10 mg (w/v) of the soluble starch that was dissolved into 1.0 ml of the D_2O solution. The reaction products were analyzed by ^{1}H-NMR at different reaction times (Fig. 3). During the first 5 to 30 min, a chemical shift around 5.2 ppm ($J = 3.8$ Hz), due to the α-anomer of the products, was observed. However, there was no chemical shift around 4.65 ppm ($J = 8.0$ Hz), due to the β-anomer of the products.

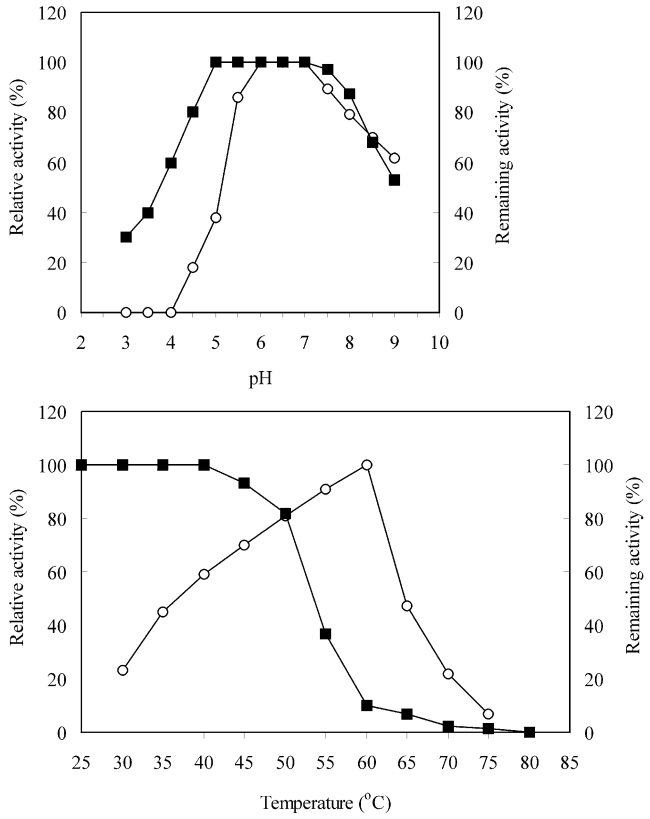

Fig. 2. The pH and temperature effects on the activity and stability of the maltose-forming α-amylase. ■: remaining activity; ○: relative activity. For pH stability, the remaining activity was measured at 50°C and pH 6.0 for 10 min after incubation of the purified maltose-forming α-amylase at different pH for 24 h. For the heat stability, the remaining activity was measured at 50°C and pH 6.0 for 10 min after treatment of the enzyme at different temperatures for 15 min.

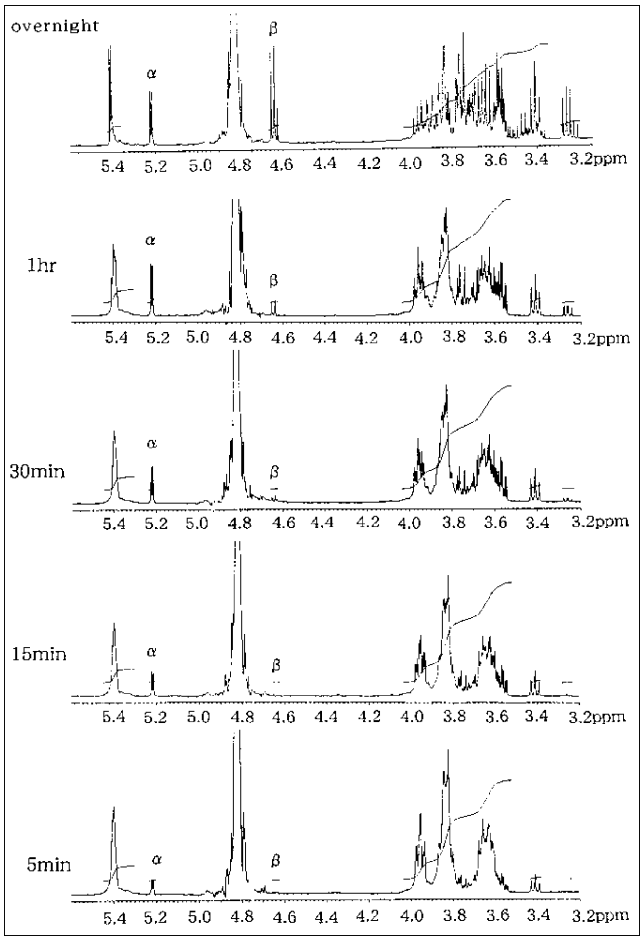

Fig. 3. ^{1}H-NMR of the products from soluble starch hydrolyzed with the maltose-forming α-amylase. The reaction was carried out by incubating the mixture of 10 mg soluble starch that was dissolved into 1 ml of D_2O and 13 units of the enzyme at ambient temperature.

However, within 1 hr, β-anomer of the products was observed. The ratio of α-anomer/β-anomer decreased during that time, due to mutarotation (α/β = 1/0.33 for a 1 h reaction time, and 1/1.78 for overnight-reaction time).

Action pattern on soluble starch One unit of the purified enzyme was added to the same volume of the 1% (w/v) soluble starch solution, and the enzyme reaction was carried out at 30°C and pH 6.0. The products of this reaction were analyzed during several time intervals by HPLC, and the percentages of the different products were determined (Table 2). Oligosaccharides in the range of maltose to maltopentose are the first hydrolytic products to be released; therefore, maltose increases in all of the digests. After a 72 h incubation, the major product from soluble starch was maltose, which represented about 75% of the total amount of all of the products combined. The only time that we noticed the appearance of a small amount of glucose that did not exceed 11% incubation was after a long incubation (more than 24 h). When 13 units of enzyme were reacted, the amount of produced maltose was about 90%, as determined by HPLC. This result was confirmed by TLC (Fig. 4A) This hydrolytic profile indicates a mechanism of action that is different than the classical α-amylase patterns (Doyle *et al*., 1989). On other hand, the percentage of produced maltose (75%) is higher when compared to other maltose-forming amylases that were previously reported (Wako *et al*., 1978; Hidaka and Adachi, 1980; Doyle *et al*., 1989; Collins *et al*., 1993; Doyle *et al*., 1998).

Action pattern on some maltooligosaccharides One unit of the purified enzyme was added to the same volume of 2% (w/v) solution of different maltooligosaccharides (from G2 to G6). The enzyme reactions were carried out at 30°C and pH 6.0 for different time intervals. This enzyme did not act on maltose (data not shown), and it also required a long incubation period to hydrolyze maltotriose. The results of the enzyme reactions on G3, G4, G5, and G6 are illustrated in Figure 5. Detectable amounts of glucose were not observed

Table 2. Percentage of the different products from soluble starch that was progressively hydrolyzed by the maltose-forming α-amylase. One unit of the enzyme was reacted with 1% of soluble starch at 30°C, pH 6.0 and reaction products were analyzed at different time intervals.

Incubation time (h)	Oligosaccharide (%)			
	G1	G2	G3	Others (G4+G5)
0	0	0	0	0
0.25	0	28.2	31.2	40.5
0.5	0	47.8	31.8	20.4
1	0	54.6	29.8	15.6
2	0	56	29	15
4	0	58	27.5	14.5
24	8.2	75	4.7	12.1
72	11	75	2.8	11.2

for all of these maltooligosaccharides, except for the long-time incubations (more than 24 h) in which glucose appeared in small amounts when compared to the expected amount to be released. It was also remarkable for all of these digestions that glucose appeared while the amount of maltotriose was decreasing. This result led us to suggest that glucose may mainly come from the hydrolysis of maltotriose. However, the K_m values of this enzyme towards maltohexose, maltopentaose, malototetrose, and maltotriose were 26.7, 30.1, 20.5, and 357 mM, respectively. This low affinity to maltotriose is one of the main differences between this enzyme and the one from *P. expansum*, which was reported to have a high affinity to maltotriose that leads to the high production of maltose (Doyle *et al*., 1989).

Action pattern on maltotriose This maltose-forming α-amylase hydrolyses G4, G5, and G6 as rapidly as the reaction begins; however, it hydrolyses G3 slower. Detectable amounts of maltose were observed after a 1-h incubation; glucose only appeared after more than 4 h. After a 24-h incubation, the amount of maltose and Glucose were found to be 62% and 8%, respectively (Fig. 5) (G1/G2 ratio was 0.13). The G1/G2 ratio from the hydrolysis of maltotriose by this enzyme was smaller when compared to other maltose-forming α-amylases (Doyle *et al*., 1999). This may explain the higher amount of maltose that is produced by this enzyme. Doyle *et al*. (1989) explained the formation of the very high maltose yield by the *P. expansum* system, the significance of its high affinity for maltotriose. The opposite appears to be true in the case of our enzyme system; the low affinity for maltotriose seems to contribute to both high maltose yields and the low amount of glucose.

The amount of glucose that is released from maltotriose was unexpected. The hydrolysis of one mole of maltotriose produced one mole of glucose as well as one mole of maltose; however, in this case, the enzyme hydrolyzed maltotriose to produce maltose four times more than glucose (mole/mole). The transglycosylation event is postulated to be the major multimolecular mechanism that contributes to maltotriose degradation by our enzyme system.

Two mechanisms of transglycosylation can explain the production of four times more maltose than glucose from maltotriose (Fig. 6). This enzyme catalyses two

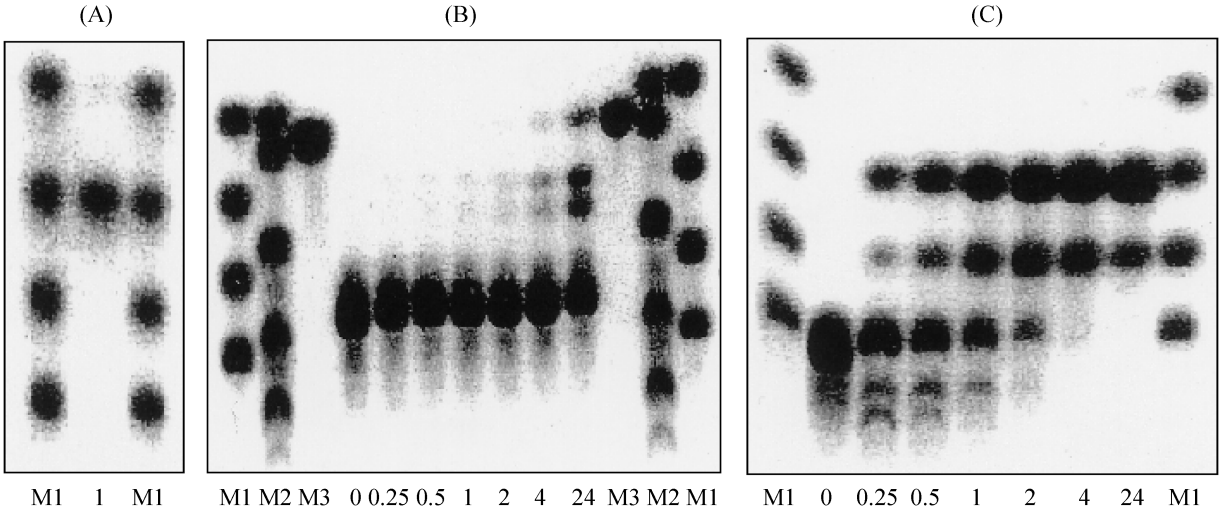

Fig. 4. TLC of the different products of the maltose-forming α-amylase when acting on soluble starch (A), fructosyl-maltotrioside (B) and maltotetraose (C). (A) Final products from 1% starch after a 24-h hydrolysis with 13 units of purified enzyme at 30°C, pH 6.0. M_1: (a mixture of G1, G2, G3, and G4); 1: spot of the enzyme reaction mixture. (B) Different products from 2% fructosyl-maltotrioside after hydrolysis with 1 unit of purified enzyme at 30°C, pH 6.0 for different incubation times (0 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, and 24 h). M_1: (a mixture of G1, G2, G3, and G4), M_2: (a mixture of F1, F2, F3, F4, F5, and F6), M_3: sucrose. (C) Different products from 2% maltotetraose after hydrolysis with 1 unit of purified enzyme at 30°C, pH 6.0 for different incubation times (0 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, and 24 h).

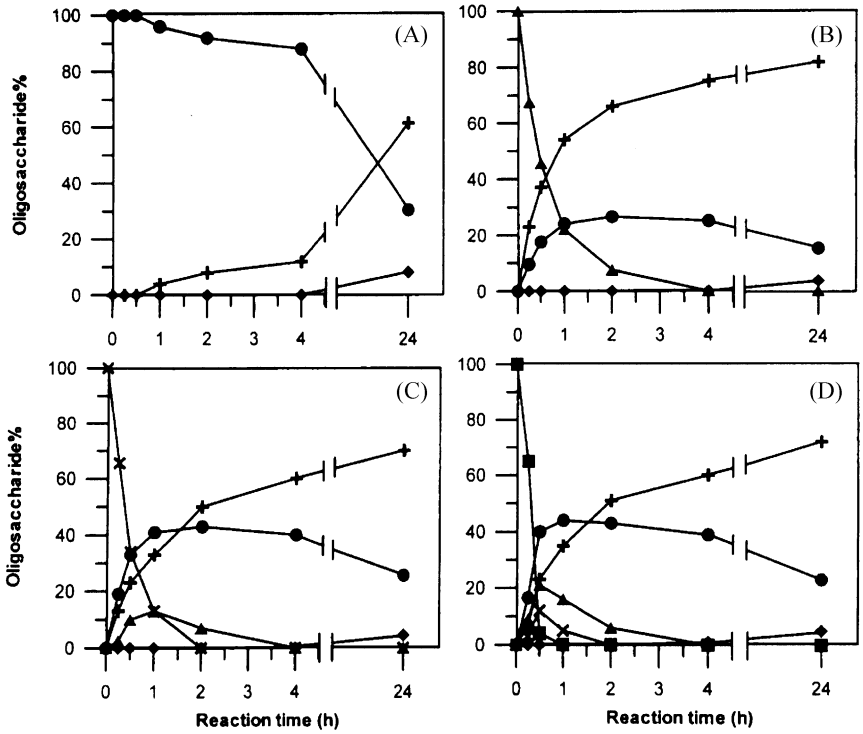

Fig. 5. Percentages of the different products from the hydrolysis of various maltooligosaccharides by the maltose-forming α-amylase. (A), (B), (C), and (D) represent, respectively, the hydrolysis of maltotriose, maltotetrose, maltopentose, and maltohexose by the maltose-forming α-amylase. G1: ◆; G2: ●; G3: ▲; G4: ■; G5: +; G6: ×. One unit of the enzyme was acted on a 2% solution of each maltooligosaccharide. The enzyme reaction was carried out at 30°C, pH 6.0 for different time intervals.

transglycosylation reactions (I and II) to produce, respectively, maltotetrose and maltopentose as transient products. These transient products are undetected in the reaction mixture since their hydrolyses occur more rapidly than maltotriose. To produce four times more maltose than glucose (mole/mole), reactions I and II should occur without predominance (reaction I : reaction II = 50 : 50). The α-amylase from *Streptomyces praexos* shows a similar action pattern for the hydrolysis of maltotriose (Suganuma *et al*., 1980); however, the probability of two transfer reactions (reactions I and II) differs from our case. The α-amylase from *Streptomyces praexo* catalyses the two reactions with a rate of (94 : 6).

Action Pattern on fructosyl-maltotrioside One unit of the purified enzyme was added to the same volume of 2% (w/v) of the fructosyl-maltotrioside solution. The enzyme reaction was carried out at 30°C and pH 6.0 for different time intervals. The products of this reaction were analyzed by TLC (Fig. 4B). The products were sucrose, maltose, and fructosyl-maltoside. However, glucose, fructose, and maltotriose were not produced by this α-amylase. The action pattern of this enzyme can be explained by the reactions III and IV (Fig. 6). Reaction III is a simple hydrolysis of fructosyl-maltotrioside to produce maltose and sucrose. According to reaction IV, transglycosylation could be a determinant process to produce maltose and fructosyl-maltoside from fructosyl-maltoteraoside that represents a transient product in this reaction.

Action pattern on maltotetrose The implication of the transglycosylation reaction in the action pattern of this maltose-forming enzyme was clearly identified by analyzing the products that were released from the hydrolysis of maltotetrose by TLC (Fig. 4C). After incubation of 1 unit of the purified enzyme with the same volume of 2% solution of maltotetrose, we noticed the production of maltose, maltotriose, maltopentose, and maltohexose. The presence of maltopentose and maltohexose, which were found to be transient products, confirms that the transfer reactions are catalyzed by this enzyme. Two possible pathways are also possible for the hydrolysis of maltotetrose (reactions V and VI) (Fig. 6).

The action pattern of this enzyme on maltotetrose seems to be unique and different from the other maltogenic α-amylase that was previously reported. Collins *et al.* demonstrated the combined roles of transglycosylation and condensation in the hydrolysis of maltotetrose by the α-amylase from *T. curvata* (Collins *et al*., 1993). The α-amylase of porcine pancreas also showed a different hydrolysis pathway of maltotetrose in which the condensation reaction played a major role (Robyt and French, 1970).

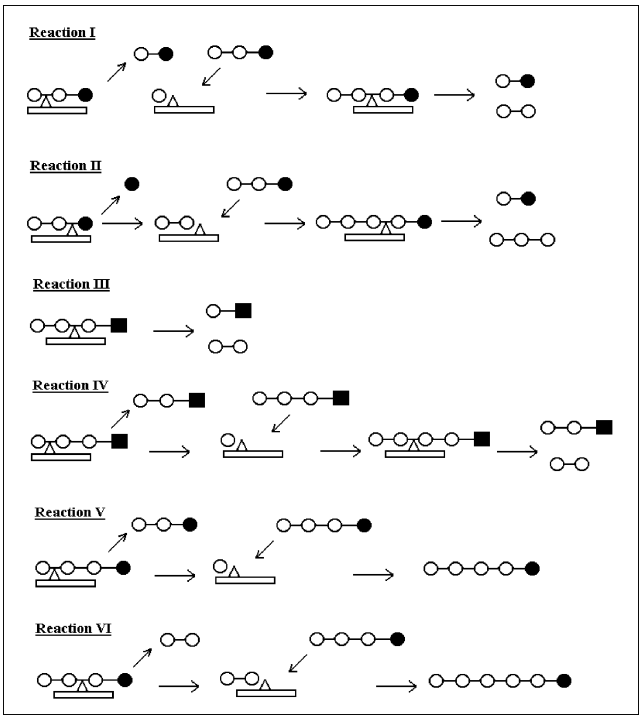

Fig. 6. Schematic representation of the possible action pattern o the maltose-forming α-amylase on maltotriose (Reactions I anc II), fructosyl-maltotrioside (Reactions III and IV) anc maltotetraose (Reactions V and VI). ○: glucose unit, ●: reducing end of the original substrate molecule, ■ : fructose unit

Possible use of the enzyme in bread-bakery process This maltose-producing α-amylase was tested for its application in the bread-bakery process. As a result, this enzyme was found to have a good impact on bread characteristics, such as specific volume and softness when compared to the bread without the enzyme addition. The bread baked after the addition of enzyme showed a relatively higher volume (2412 ml) and lower weight (383.4 g) when compared to the one without the enzyme addition (2371 ml and 385.3 g). Therefore, the specific volume of the bread that was baked after the enzyme addition was 6.29 ml/g. In addition, this maltose-producing α-amylase eventually showed a great benefit in retaining the softness of the bread. Table 3 shows the evolution of the bread softness after 7 d of conservation. The control bread, without the enzyme addition, increased in hardness faster than the bread with the added enzyme. The higher values of the resistance force in Table 3 indicate harder bread. Accordingly, the addition of maltose-producing α-amylase kept the bread softer, even after 7 days.

In conclusion, the maltose-forming α-amylase from *Streptomyces* sp. that was reported in this paper was interesting for many reasons.

(1) It produces a high yield of maltose that represents up to 90% after complete hydrolysis of the starch.

(2) The conversion of starch and maltooligosaccharides, primarily to maltose, without synchronous glucose production.

(3) The action pattern on maltooligosaccharides seems to be unique, involving transglycosylation reactions in the same time as the unimolecular hydrolysis system.

(4) The enzyme showed interesting results regarding its possible utilization in the bread-bakery process, giving high specific volume and softness to the bread, even after days of conservation.

Table 3. Softness of the bread during the time after the addition of the maltose-producing α-amylase

	Softness of the bread (g/cm^2)		
	After 1 day	After 3 days	After 7 days
Control*	181	293	373
G2 α-amylase**	156	252	322

*Without enzyme
**With maltose-producing α-amylase

References

Campbell, L. L. (1955) Purification and properties of an α-amylase from facultative thermophilic bacteria. *Arch. Biochem. Biophys.* **54**, 154-161.

Collins, B. S., Kelly, C. T., Fogarty, W. M. and Doyle, E. M. (1993) The high maltose-producing α-amylase of the thermophilic actinomycete *Thermomonospora curvata. Appl. Microbiol. Biotechnol.* **39**, 31-51.

Doyle, E. M., Kelly, C. T. and Fogarty W. M. (1989) The high maltose-producing α-amylase of *Penicillium expansum. Appl. Microbiol. Biotechnol.* **30**, 492-496.

Doyle, E. M., Noone, A. M., Kelly, C. T. and Fogarty, W. M. (1999) Comparison of the action pattern of two high maltose-forming α-amylase on linear maltooligosaccharides. *Enzyme Microb. Technol.* **25**, 330-335.

Doyle, E. M., Noone, A. M., Kelly, C. T., Quigley, T. A. and Fogarty, W. M. (1998) Mechanisms of action of the maltogenic α-amylase of *Byssochlamys fulva. Enzyme Microb. Technol.* **22**, 612-616.

Fogarty, W. M. (1983) Microbial amylases; in *Microbial enzyme and biotechnology*, Fogarty, W. M. (ed.), pp. 1-92, Applied Science Publishers, London.

Goldberg, J. D. and Edwards, C. (1990) Purification and characterization of an extracellular amylase from a thermophilic streptomycete. *Appl. Bacteriol.* **69**, 712-717.

Hidaka, H. and Adachi, T. (1980) Studies on the α-amylase from *streptomyces hygroscopicus* SF 1084; in *Mechanisms of saccharide polymerisation and depolymerisation*, Marshall, J. J. (ed.), pp. 101-108, Academic Press, New York, New York.

Obi, S. K. C. and Odibo, F. J. C. (1984) Some properties of a highly thermostable α-amylase from *Thermoactinomyces* sp. *Can. J. Microbiol.* **30**, 780-785.

Robyt, J. and French, D. (1970) Action pattern of porcine pancreatic α-amylase in relationship to the substrate binding site of the enzyme. *J. Biol. Chem.* **245**, 3917-3927.

Somogyi, M. (1952) Notes on sugar determination. *J. Biol. Chem.* **195**, 19-23.

Suganuma, T., Mizukami, T., Moori, K., Ohnishi, M. and Hiromi, K. (1980) Studies on the action pattern of an α-amylase from *Streptomyces praecox* NA-273. *J. Biochem.* **88**, 131-138.

Wako, K., Takahashi, C., Hashimoto, S. and Kanaeda, J. (1978) Studies on maltotriose- and maltose-forming amylases from *Streptomyces*. *J. Jpn. Soc. Starch. Sci.* **25**, 155-161.

Weber, K. and Obsorn, M. (1975) Proteins and sodium dodecylsulphate: molecular weight determination on polyacrylamide gels and related procedures; in *The proteins*, Vol. 1, Neurath, H., Hill, R. L. and Border, C. (eds.), pp. 179-233, Academic Press, New York, New York.