0273-2289/91/3002—0193$02.00

HPLC Study of Starch Hydrolysis Products Obtained with α-Amylase from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*

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Received July 8, 1990; Accepted September 6, 1990

ABSTRACT

Two bacterial α-amylases from new industrial strains were studied: α-amylase from *Bacillus amyloliquefaciens* CCM 3502 (Czechoslovak) and thermostable α-amylase from *Bacillus licheniformis* 44MB82 (Bulgarian). The thermostable enzyme hydrolyzed starch mainly to dextrins, and after 1 h, 30% of the products were oligosaccharides. The *B. amyloliquefaciens* enzyme produced more maltooligosaccharides than the first enzyme (*B. licheniformis*). Within 1 h, up to 80% of the substrate were hydrolyzed, giving different spectrum of oligosaccharides in comparison with the thermostable one.

Index Entries: α-Amylase; *Bacillus amyloliquefaciens; Bacillus licheniformis;* HPLC; soluble starch; maltooligosaccharides.

INTRODUCTION

α-Amylase is widely used as a simple and inexpensive means to carry out the first step of depolymerization of starch.

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Thermostability of α-amylases is advantageous since the hydrolysis occurs above the gelatinization temperature, i.e., 95–105°C. A short treatment at a high temperature with a thermostable liquefying α-amylase will convert starch into smaller more soluble dextrins that can be further processed at a lower temperature by different amylases.

Two α-amylases were used in this study: a thermostable α-amylase from *Bacillus licheniformis* 44MB82 (*1*) and α-amylase from *Bacillus amyloliquefaciens* CCM 3502 (*2*). The aim was to compare the quantities of oligosaccharides produced from diluted soluble starch by low amounts of the two different α-amylases.

MATERIALS AND METHODS

Sources of Enzymes

The producer of α-amylase (T optimum 60°C) *B. amyloliquefaciens* CCM 3502 was acquired at the Institute of Microbiology, CSAV, Prague and was registered as a new Czechoslovak industrial strain (*2*). The fermentation liquid, after a cultivation of the strain on complex starch medium, was used for isolation and purification of the enzyme. Lyophilized powder was employed for further experiments. The thermostable α-amylase (T optimum 90°C) was isolated from *B. licheniformis* 44MB82, which was obtained after NTG treatment of *B. licheniformis* 44MB80 in the Institute of Microbiology, Bulg. Acad. Sci., Sofia, and was registered as a new Bulgarian industrial strain (*1*). The enzyme was characterized, partially purified, and concentrated to enzyme activity of 50,000 U/mL (*3*).

Conditions of Starch Hydrolysis

A liquid concentrate of thermostable α-amylase from *B. licheniformis* 44MB82 (*1,2*) and a lyophilized powder of α-amylase from *B. amyloliquefaciens* (*3*), dissolved in 0.02*M* phosphate buffer, pH 6.3 were used. The substrate for the thermostable enzyme, soluble starch (prepared by the method of Leulier), was dissolved in 0.066*M* phosphate buffer, pH 7 to obtain a final concentration of 20.0 mg mL^{-1} (w/v). The reaction mixture consisted of 5 mL of a soluble starch solution and of 0.5 mL related to the thermostable enzyme (4 U mL^{-1}). All data were recalculated on the original concentration of soluble starch (20 mg mL^{-1}).

The substrate solution for *B. amyloliquefaciens* was prepared from soluble starch dissolved in distilled water. The final concentration was 20 mg mL^{-1}. The initial enzyme concentrations were 4 U mL^{-1} (thermostable α-amylase) and 0.5 DNS U mL^{-1} (*B. amyloliquefaciens* amylase). The reaction mixture contained equal vol of the starch and the enzyme solution; the final concentration of the starch was 10 mg mL^{-1}.

Enzyme Assay and Units Definition

The α-amylase activity of the thermostable enzyme was measured by the method of Pantschev et al. (*4*). One enzyme unit was equivalent to the amount of the enzyme that hydrolyzed 0.025 mg of 1% soluble starch solution to dextrins within 1 min at 30°C and pH 6.5. One DNS unit of *B. amyloliquefaciens* α-amylase was equivalent to 1 mmol of D-maltose released from 1% soluble starch solution within 3 min at 25°C, as measured by the 3,5-dinitrosalicylic acid reagent (*5*). The ratio between these two units (Pantschev et al. and Bernfeld) is about 8:1 and, therefore, similar concentrations of the two enzymes were used.

The two α-amylases have the temperature optima at 90 and 60°C at standard assay conditions (pH 6.5 and 6.3), respectively, and for these estimations the reactions proceeded for 1 h at 90 and 55°C, respectively. The hydrolysis with the thermostable enzyme was stopped with 0.1*M* NaOH. Each sample was filtered through a Millipore® ultrafiltration cell before use. When the α-amylase from *B. amyloliquefaciens* was investigated, 1-mL samples were placed in microcuvets containing 10 μL of 2*M* NaOH, centrifuged, and filtered on Separon™ microfilter-nylon before analysis.

HPLC of Start Hydrolysate

Carbohydrate analysis was performed by using a Waters Liquid Chromatograph system equipped with a differential refractometer R-400 and a pump system 1000. A Hewlett-Packard model 3393A integrator was added for quantification. The column was Separon SGX RPS 4×250 mm, particle size of 7 μm (Tessek Ltd., Czechoslovakia). Degassed, double-distilled water, served as the mobile phase, the flow rate being 0.7 mL min^{-1} at ambient temperature. The injection vol of either hydrolysate was 20 μL. D-Glucose, maltose, and maltooligosaccharides from maltotriose to maltoheptaose (Boehringer) was used as the standards in concentrations from 0.5 to 1.5 mg mL^{-1}. The contents of reducing substances were determined by the method of Somogyi (*6*), using maltose as the standard.

RESULTS

Concentrations of maltooligosaccharides and D-glucose obtained with the two enzymes are shown in Figs. 1–7.

Five major maltooligosaccharides (from maltose to maltohexaose) and D-glucose were detected when the thermostable α-amylase from *B. licheniformis* was examined. According to Fig. 8 summarizing all seven measured sugars, 26% of the substrate was hydrolyzed to glucose and oligosaccharides after 15 min. The rest were dextrins but their hydrolysis proceeded at a low rate.

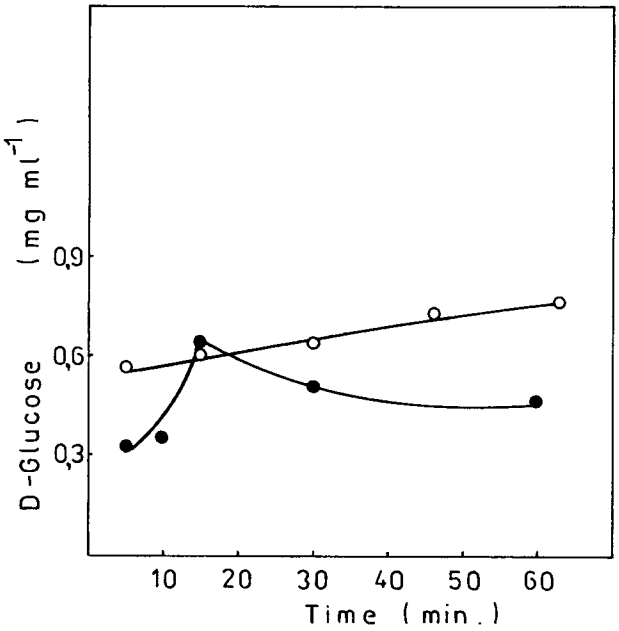

Fig. 1. Time-course of glucose (DP1) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *Bacillus amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

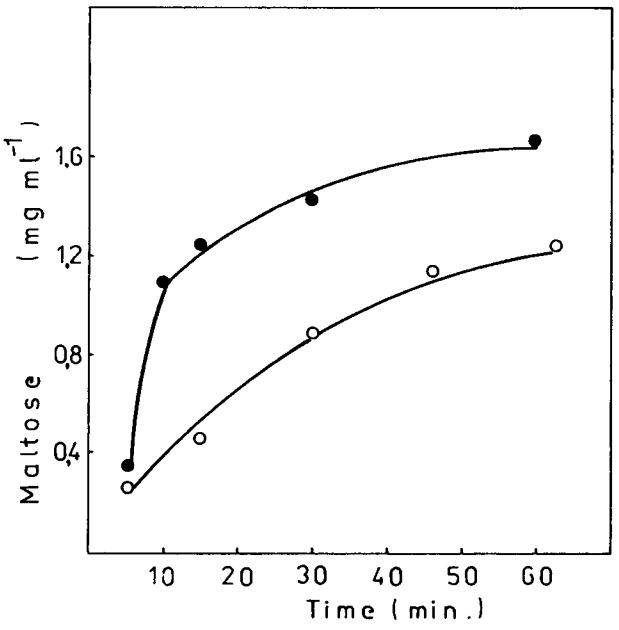

Fig. 2. Time-course of maltose (DP2) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

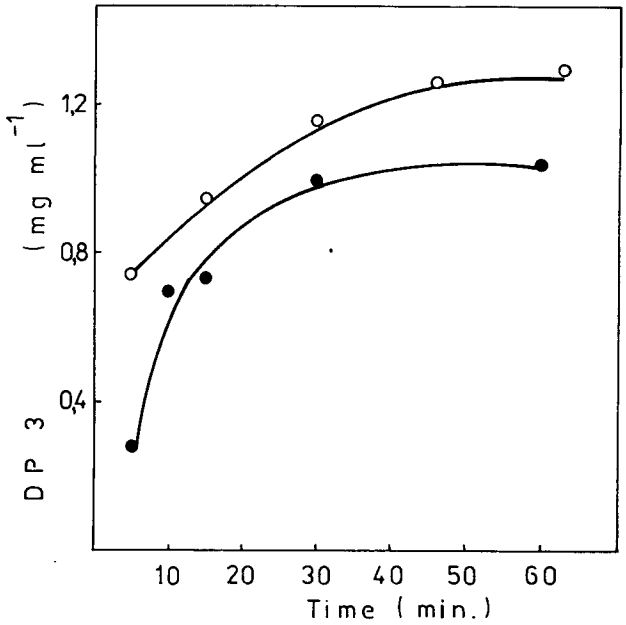

Fig. 3. Time-course of maltotriose (DP3) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

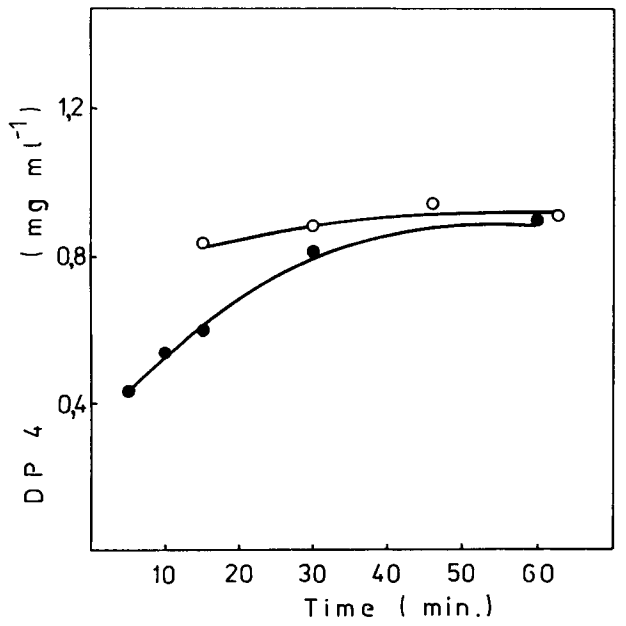

Fig. 4. Time-course of maltotetraose (DP4) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

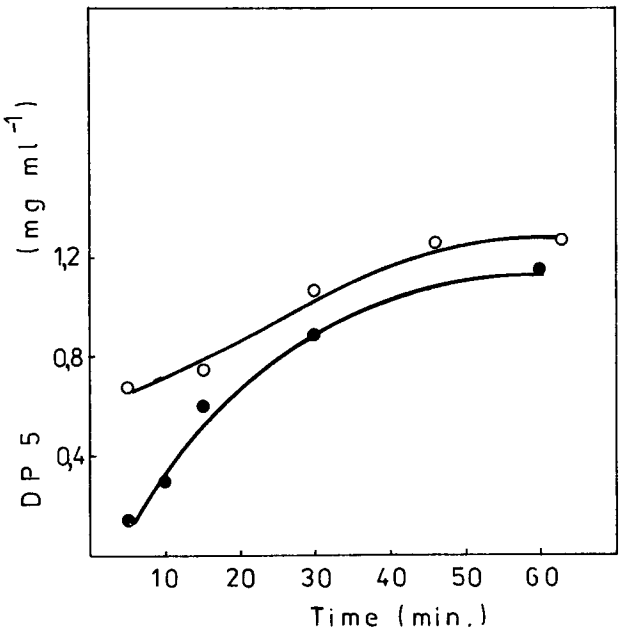

Fig. 5. Time-course of maltopentaose glucose (DP5) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

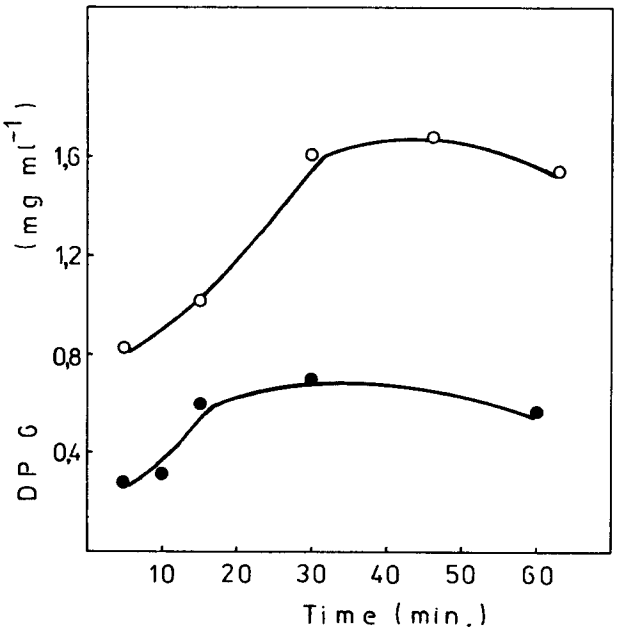

Fig. 6. Time-course of maltohexaose (DP6) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

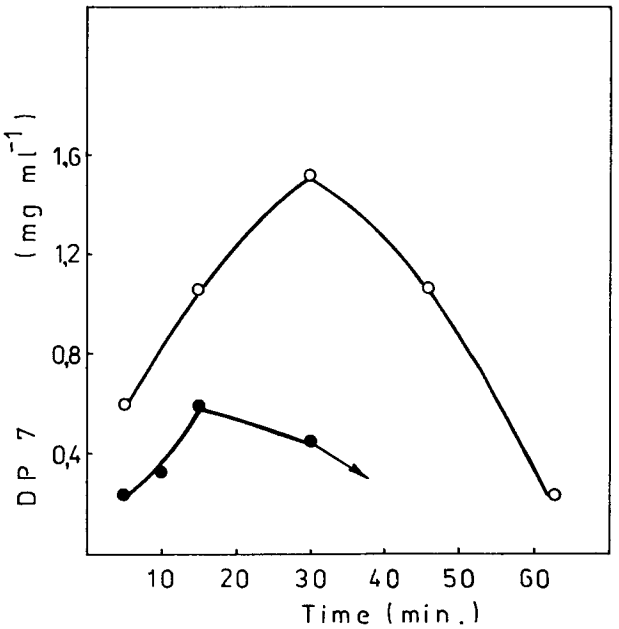

Fig. 7. Time-course of maltoheptaose (DP7) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

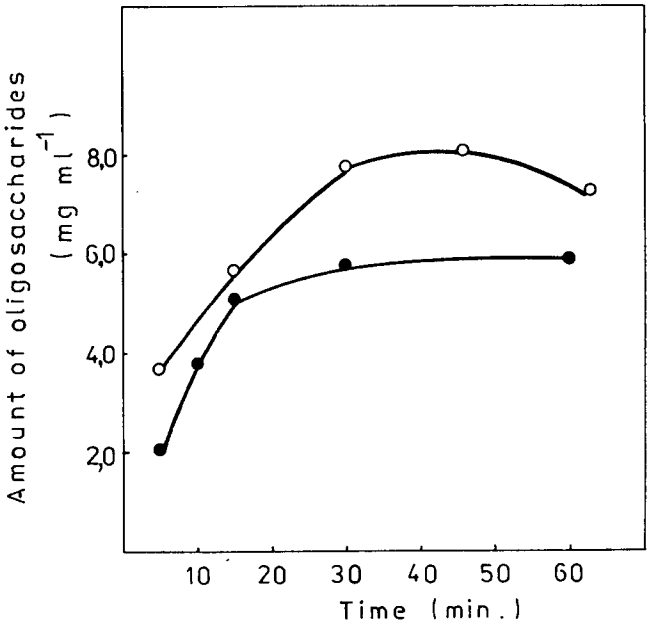

Fig. 8. Time-course of total oligosaccharides (DP1–DP7) concentration measured by HPLC in a soluble starch solution hydrolyzed by two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

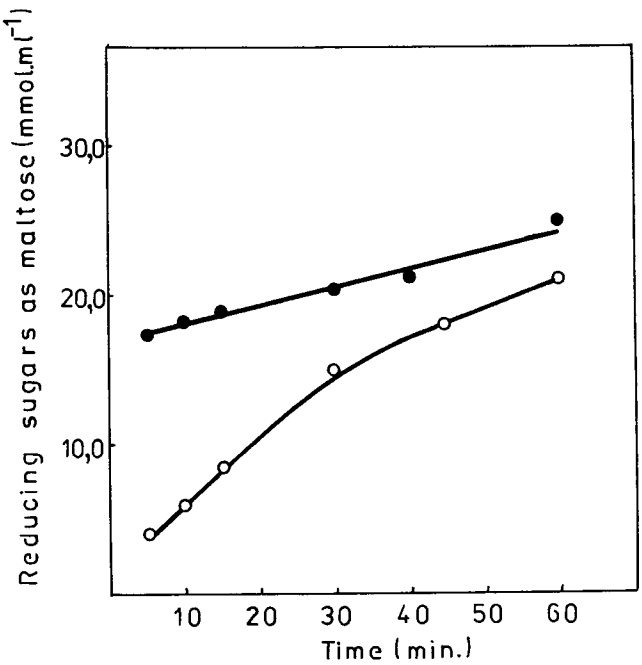

Fig. 9. Accumulation of reducing sugars measured by DNS method in a reaction mixture of soluble starch and two α-amylases. Open symbols: α-amylase from *B. amyloliquefaciens;* closed symbols: α-amylase from *B. licheniformis.*

The total amount of the low-mol wt products was 5.9 mg mL^{-1} after a 60-min hydrolysis at 90°C, i.e., 29.5% of the soluble starch had been hydrolyzed to small oligomers. The reducing value (maltose) was 25 mmol mL^{-1} (Fig. 9) (8.57 mg mL^{-1}). The main low-mol wt product of the process was maltose (Fig. 2) and its concentration represented 28.8% of the total amount of maltosugars. The maltose level reached 1.7 mg mL^{-1} after 1 h enzyme action (8.5% of the total substrate). A similar mode of action was observed also for the products with polymerization degrees 3 and 5 (Figs. 3 and 5), but their final concentration was lower (1–1.2 mg mL^{-1}). A relatively high amount of maltotetraose was also produced and its concentration reached 0.9 mg mL^{-1}. DP7 was obtained during the first steps of the reaction but was hydrolyzed during the process, and could not be detected in the hydrolysates after 30 min. The content of D-glucose (Fig. 1) reached a value of 0.45 mg mL^{-1}. The product distribution showed a slow formation of small chain oligomers early in the hydrolysis, this α-amylase was thus of the liquefying type. The dominant oligosaccharides were maltose, DP3, and DP5. These results are in agreement with those of Dobreva et al. (*8*), obtained for the same enzyme previously.

When comparing the action pattern of the thermostable α-amylase from *B. licheniformis* with that of the enzyme from *B. amyloliquefaciens* on

soluble starch, the enzymes differed in several points. The α-amylase from *B. amyloliquefaciens* behaved as other α-amylases, producing only small amounts of maltose (*9*).

The main low-mol wt products after 1 h of degradation were maltohexaose, DP5, and DP3 (Figs. 3, 5, and 6). These oligomers represented 47.5% (3.8 mg mL^{-1}) of the low-mol wt products. The concentration of DP6 was 1.7 mg mL^{-1} (21% of all oligosaccharides), but its time profile showed a declining tendency (Fig. 6). The concentration of DP4 was similar to those obtained with the thermostable enzyme from 20 mg mL^{-1} soluble starch. DP7 was also detected during the first hour. Its concentration reached a high value (1.5 mg mL^{-1}) and then declined with time (Fig. 7). The final total amount of D-glucose and oligomers obtained by the action of the enzyme (Fig. 8) showed that 80% of the substrate present at a concentration of 10 mg mL^{-1} was hydrolyzed within 1 h to low-mol wt products. The reducing value (maltose) was 21.01 mmol mL^{-1} (7.185 mg mL^{-1}) (Fig. 9). The analysis of the hydrolytic products showed the α-amylase to be an enzyme of the saccharigenic type. The amounts of the products decreased in the order: maltohexaose and maltopentaose, maltose, DP3, D-glucose, and DP4.

DISCUSSION

The specificity of an α-amylase depends on the bacterial strain and, thus, each α-amylase gives a characteristic distribution of oligosaccharides by splitting α-glucan. This may reflect differences in numbers and substrate affinities of the subunits at the enzyme active center (*10*).

The amino acid sequence of *B. licheniformis* α-amylase has an 80% homology with the α-amylase from *B. amyloliquefaciens* (*11*). According to our preliminary results, both α-amylases have a similar mol wt of about 58–60 kDa. However, the two enzymes used in this study differ under standard conditions in their optimal temperatures of action (90 and 60°C), in their thermostability (K_M) values (the thermostable enzyme, 0.90 mg mL^{-1}; the other enzyme, 5.93 mg mL^{-1}), and in the composition of their low-mol wt products. The thermostable α-amylase from *B. licheniformis* strain 44MB82 hydrolyzes starch mainly to dextrins, and the enzyme can be concluded to be of the liquefying type. In contrast, *B. amyloliquefaciens* α-amylase hydrolyzes this substrate to maltooligosaccharides, and could be used as a saccharifying enzyme.

An important distinction between the two enzymes was found when the resulting amounts of maltose were compared. The α-amylase from *B. licheniformis* strain produced 2.5 times more maltose during the first 15 min of reaction. A difference was also found in the distribution of the released oligosaccharides. Maltose, maltopentaose, and DP3 were dominant

oligomers produced by the hydrolysis of starch with the thermostable enzyme. The *B. amyloliquefaciens* α-amylase liberated mainly maltohexaose and maltopentaose.

REFERENCES

1. Beschkov, M., Emanuilova, E., Grigorova, R., Kosturkova, P., Dobreva, E., Tonkova, A., Schivarova, N., and Kamenova, K. Bulg. Patent No. 37765 (09.05.86)
2. Pazlarová, J., Fencl, Z., and Tichý, P. Czechoslovak Patent No. 217313 (01.08.85)
3. Dobreva, E., Emanuilova, E., Pantscheva, V., Philopova, Ts., and Pantschev, S. (1987), *Biotechnol. Biotechnic.* **2,** 30–32.
4. Pantschev, Ch., Klenz, G., and Häfner, B. (1981), *Lebensmittelindustrie* **28,** 71–74.
5. Bernfeld, P. (1955), *Methods Enzymol.* **1,** 149–158.
6. Somogyi, M. (1952), *J. Biol. Chem.* **195,** 19–23.
7. Dobreva, E. and Ivanova, V. (1989), *Acta Biotechnol.* **9,** 577–582.
8. Chiang, J., Alter, J., and Sternberg-Elkhart, M. (1979), *Stärke* **31,** 86–92.
9. Mercier, Chr. and Colonna, P. (1988), *Biofutur* **11,** 55–60.
10. Yuuki, T., Nomura, T., Tezuka, H., Tsuboi, A., Yamagata, H., Tsukagoshi, N., and Udaka, S. (1985), *J. Biochem. (Tokyo)* **98,** 1147–1156.
11. Tomazic, S. and Klibanov, A. M. (1988), *J. Biol. Chem.* **263,** 3092–2096.