Kor. J. Appl. Microbiol. Biotech.
Vol. 18, No. 5, 484-489 (1990)

Partial Purification and Properties of Non-specific β-fructofuranosidase Produced by *Bacillus subtilis*

Song, Geun-Seoup and Tai-Boong Uhm*

Department of Food Technology, Chonbuk National University, Chonju 560-756, Korea

*Bacillus subtilis*가 생산하는 비특이적 β-fructofuranosidase의 부분정제 및 특성

송근섭 · 엄태붕*

전북대학교 식품공학과

An intracellular inulase (β-fructofuranoside fructohydrolase, EC 3.2.1.26) from *Bacillus subtilis* has been partially purified and its mode of action and general properties were studied. The enzyme had an apparent molecular weight of 49,000 as estimated by gel filtration and its pI point was 5.2. Substrate concentration studies showed an apparent K_m of 10 mM for sucrose and of 18 mM for raffinose. The enzyme was an acid-labile protein with a pH optimum of 6.6. The optimum temperature was 50°C. The enzyme acts on straight chain oligo- and poly-fructosides of the inulin series via a exo-wise cleavage mechanism, as well as on sucrose.

Inulin, a reserve carbohydrate in plants of Jerusalem artichoke, chicory, and dahlias, is a linear β-2,1 linked fructose polymer terminated by a sucrose unit residue (1). It can be hydrolyzed by acids or enzymes to produce fructose. But the enzyme hydrolysis is preferred because of a lower by-product formation than that by acids (2). As an attractive method to produce the "Ultra High Fructose Glucose Syrups" (UHFGS) by the hydrolysis of inulin enzymatically, attention has been concentrated on the potential industrial application of microbial inulases. Microbial inulases have been described mainly from yeasts such as *Kluyveromyces* (3-5), *Candida*(6) and *Debaryomyces* species (7, 8), but fungal inulases from *Aspergillus* (9, 10) and *Penicillium* species (11) have been reported. However, inulases have been found only occasionally in bacteria (12, 13).

Recently, we isolated a bacterium which has never been described as an inulin hydrolyzing microorganism previously and identified this as a *Bacillus subtilis* (14). Therefore, we have tried to purify the inulase from *Bacillus subtilis* for better understanding of the enzymatic properties.

This paper describes the partial purification and some of kinetic and action mode of non-specific β-fructofuranosidase (EC 3.2.1.26), produced by *Bacillus subtilis.*

Key words: Inulin, fructose, inulase purification, enzyme characterization, reaction mechanism, *Bacillus subtilis*
*Corresponding author

Materials and Methods

Production and purification of *B. subtilis* inulase

Bacillus subtilis JU-70 was isolated originally from decayed artichoke tubers (14). The complex medium was described by Uhm *et al.* (14). Freeze dried JU-70 was initially subcultured in 50 m*l* of the complex medium with vigorous shaking for 8 h at 40°C. Inulase was produced by incubation of 2 m*l* of this culture into a 250 m*l* Erlenmeyer flask containing 100 m*l* of the complex meidum and incubated at 40°C and 200 rev. min^{-1}. Cells were then harvested after 8 h by cen-

trifugation at 2,500g for 20 min.

The paste was frozen and stored at −20°C until use. Frozen cells paste (6g) was thawed in 25 m*l* buffer A (100 mM sodium phosphate buffer pH 6.6) and then disrupted by passage through a French pressure cell at 18,000 lb fource/in². The degree of disruption was confirmed by a phase contrast microscope and no intact microorganism was detected. The resulting suspension was centrifuged at about 25,000g for 30 min. The supernatant was used as the crude extract. Subsequent procedures were performed at 4°C unless otherwise stated.

Acetone (−60°C) was dropwise added to the crude extract with constant stirring. Acetone concentrations ranging from 35 to 60% (v/v) were optimum for the precipitation of the inulase. Active fractions were collected by centrifugation at 22,000g for 15 min and then freeze-dried. Crude lyophilized inulase was dissolved in the 7 m*l* buffer A and chromatographed on Sephadex G-100 (Pharmacia) column (1.6 cm × 90 cm) at a flow rate of 5 m*l* h^{-1} cm^{-2}. Fractions of about 6 m*l* were collected. Active fractions were pooled and dialyzed against buffer B (100 mM Tris/HCl buffer, pH 7.4) overnight.

The dialyzed solution was adsorbed onto a 25 cm × 2.5 cm column of DEAE Sephacel (Pharmacia) previously equilibrated with buffer B. The column was washed with two bed volumes of buffer B before development by gradient additions of 500 mM KCl in the same buffer. The flow rate was 26 m*l* hr^{-1}. Active fractions were eluted with 0.28 M KCl (Fig. 1). Fraction having activity were pooled and dialyzed with buffer A overnight. The dialysate was then concentrated with a ultrafiltration kit (M.W. cut-off 20,000. Toyo) and stored at −20°C until use.

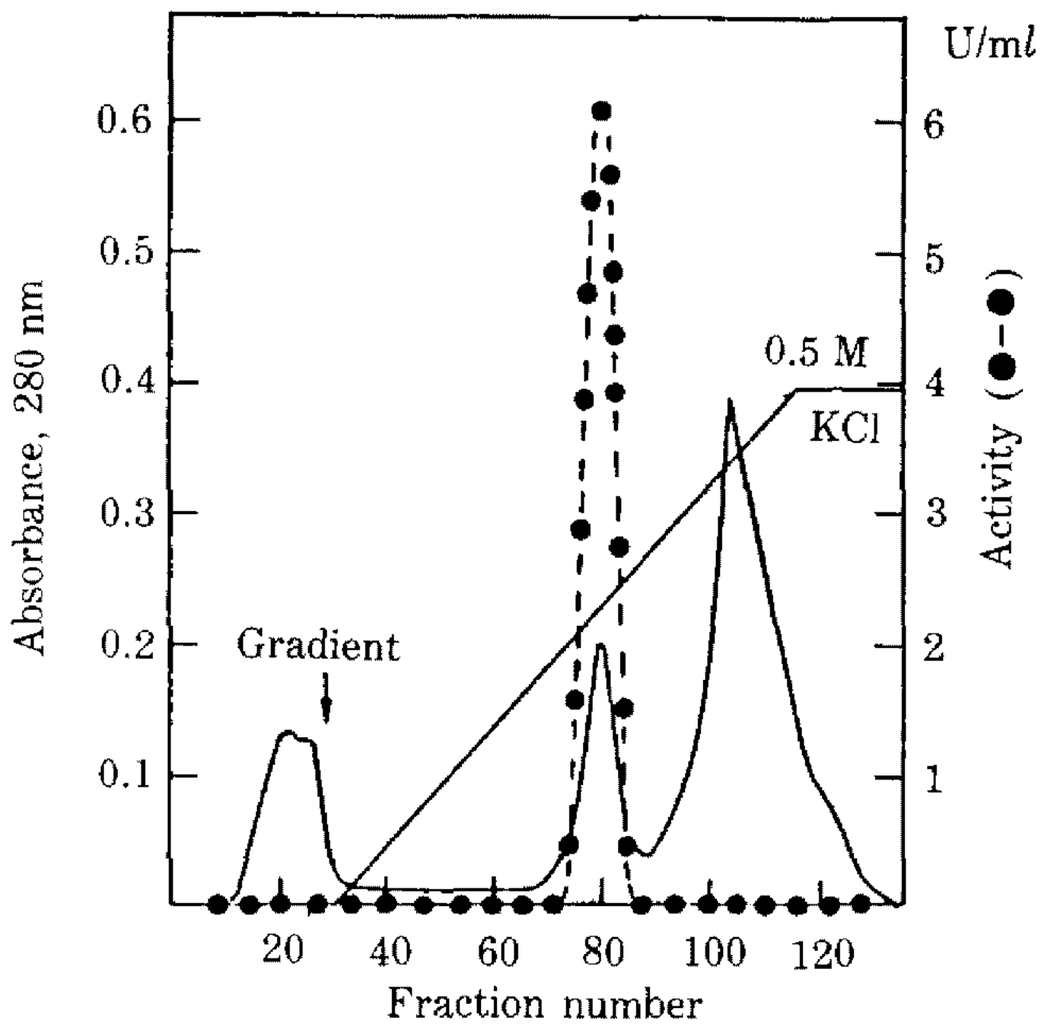

Fig. 1. DEAE-Sephacel column chromatography of inulase.

Enzyme assay

Inulase was assayed by incubating diluted enzyme solution (50 μl) at 45°C with 5% (W/V) inulin in 0.45 m*l* 100 mM sodium phosphate buffer, pH 6.6. Inulin (Sigma) was free of any significant reducing sugar by the HPLC analysis. The liberating reducing sugar was determined by 3,5-dinitrosalicylic acid method (15). One unit (U) of activity was defined as 1 μmol reducing sugar produced per minute. One unit of invertase activity was defined as 1 μmol of sucrose hydrolyzed per minute under the standard assay condition except for using 5% (w/v) sucrose as substrate. All assays were performed in triplicates.

Protein determination

Protein concentration was assayed by the method of Lowry (16) with BSA as standard.

Molecular weight estimation

The molecular weight of the enzyme was estimated by comparing its mobility on Protein Pak 125 column (7.8 mm I.D. × 30 cm, Waters) with that of known standards (17). Standard proteins (Sigma) were BSA (66,000), ovalbumin (45,000), carbonic anhydrase (29,000) and α-lactalbumin (14,200). The solvent used was 0.1 M sodium phosphate buffer, pH 6.6. The effluent was monitored at 280 nm with the flow rate of 0.5 m*l*/min. Retention time of a protein having inulase activity was compared with those of known standards.

pI determination

Samples with a protein content of 500 μg protein were dialyzed against 1% glycine solution prior to electricfocusing. Samples were applied to 1 mm thick 5% (w/v) polyacrylamide gel (245 mm × 110 mm) containing 2% (w/v) carrier ampholyte 4-6.5 (Pharmacia) according to the LKB instruction manual for analytical isoelectric focusing. The gel was focused at 25 W constant power and 10°C for 2.5 h. After focusing, the gel was cut simultaneously into 37 slices by a fractionating grid. The pH gradient was determined by measuring the pH of each slice extracted with 2 m*l* of distilled

water at 20°C. Inulase activity in the gel located by incubation of 0.3 cm gel slices in 5 m*l* 5% inulin solution at 45°C overnight.

Mode of action

Degradation mode of inulin by the enzyme at various time interval was determined using a HPLC system (Waters) fitted with the Waters Model 401 refractive index detector. Amounts of fructose produced during the enzymatic reaction were measured by injecting samples (50 μl) into a 300 mm×7.8 mm column (Aminex HPX-87P, Bio-Red) with a heating mantle (80°C). Flow rate was maintained at 0.5 m*l*/min using distilled water as the solvent system. Under the operating conditions employed, the retention times for sucrose, glucose, and fructose as standard materials were 12.81, 15.39, and 22.30 minutes, respectively.

Results

Purification of enzyme

Three purification steps were performed to purify *B. subtilis* inulase. By following the procedure of DEAE ion exchange chromatography a purification of about 40-fold could be obtained and the yield was about 50% of the original activity. Slab gel electrophoresis showed that besides the main enzymatically active protein, other proteins were still present. However, no levansucrase or inulase activity from other proteins was detected. Table 1 is a typical example of the yields and specific activities of inulase at various steps its purification from *B. subtilis*.

Molecular weight and pI determination

The molecular weight as determined by gel filtration was estimated to be 49,000. The isoelectric point as determined by analytical isoelectric focusing was 5.2.

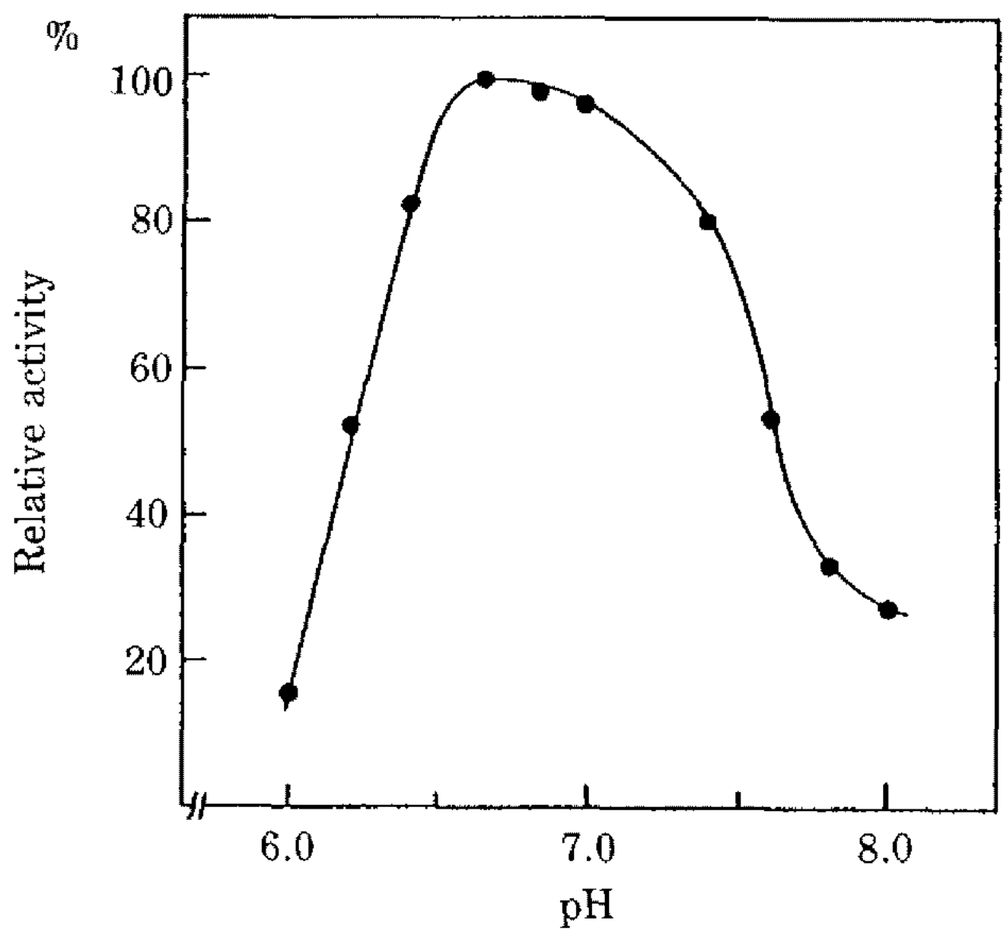

Fig. 2. Effect of pH on inulase activity.

Kinetic properties

Apparent K_m values for the two substrates, sucrose and raffinose, were determined. The K_m for sucrose was obtained by using a standard assay conditions with sucrose concentrations in the range 5 to 40 mM.

To determine the K_m for raffinose, raffinose in the range 5 to 100 mM was used as the substrate. At higher concentrations Michaelis-Menten Kinetics were not applicable.

Substrate concentration studies utilizing the Hanes equation (18) showed an apparent K_m of 10 mM for sucrose and of 18 mM for raffinose. However, we could not calculate accurate K_m value for inulin due to the no fixed molecular weight of inulin and non-Michaelis-Menten Kinetics.

pH and temperature effect

The pH optimum was assayed over the pH range

Table 1. Purification of *Bacillus subtilis* inulase

Step	Volume (m*l*)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Relative activity
French press	25	420	575	0.73	1.0
Acetone ppt. (35-60%)	7.5	279	160	1.74	2.4
Sephadex G-100	19	255	23	11.08	15.2
DEAE-Sephacel	32	218	7.3	29.86	41.0

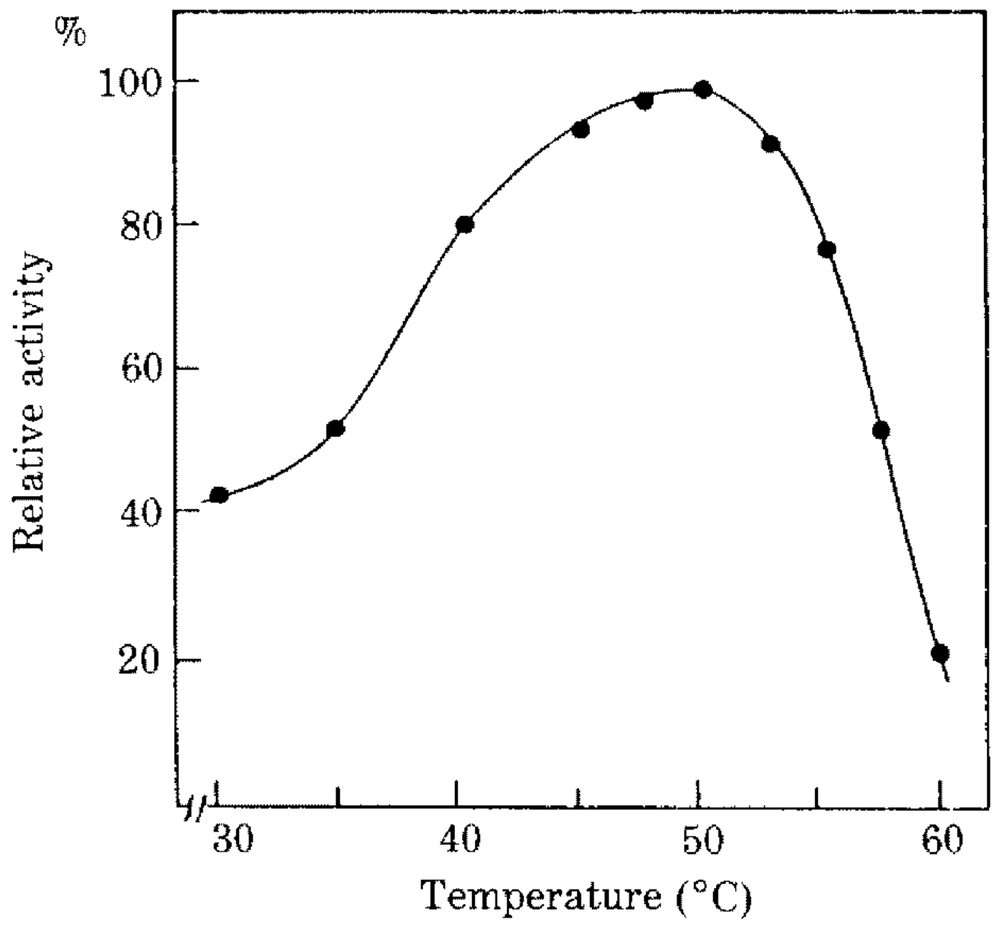

Fig. 3. Effect of temperature on inulase activity.

6.0 to 8.0 in 0.1 M sodium phosphate buffer. The optimum pH was 6.6. It fell to 18% of this value at pH 6.0 and 30% at pH 8.0 (Fig. 2). The enzyme was acid-labile below its optimum pH. However, the inulase was stable from 6.6 to 7.4 and can be stored for at least 1 month at pH 6.6 at 4°C without any detectable loss of activity.

The activity of the enzyme as a function of temperature was measured when the enzyme-substrate mixture was incubated for 10 min with the range of 30-60°C. As shown in Fig. 3, the maximum activity of the enzyme occured at 50°C. The energies of activation, Ea, obtained from the Arrhenius plot was 18 KJ mol^{-1} between 40 and 50°C.

Action mode

The products formed from inulin by the action of the inulase were analyzed by HPLC. As shown in Fig. 4, the amount of fructose increased at a constant rate during the initial phase of the reaction. Fructose was the first and only sugar produced, except that during the final stages of the hydrolysis a small amount of glucose could be detected. Considering these results, the enzyme appears to be a β-fructofuranosidase which hydrolyzes the β-2,1 linkage of inulin by stepwise liberation of fructose monomers from chain ends.

Substrate specificity

The enzyme catalyzed the hydrolysis of sucrose, raffinose and stachyose by splitting off terminal β-2,1 linked fructofuranosyl units. Relative activities toward sucrose, raffinose, and stachyose under the standard

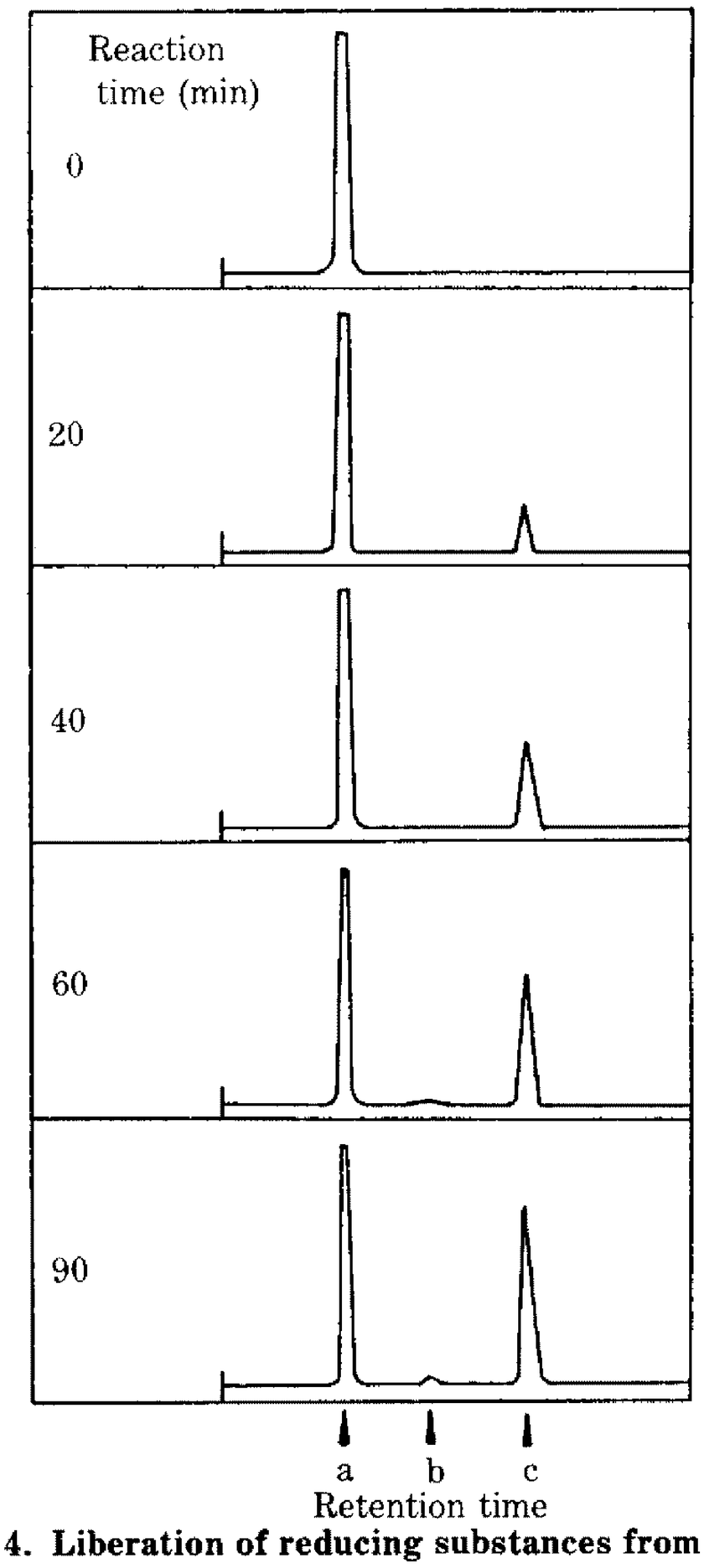

Fig. 4. Liberation of reducing substances from inulin by *B. subtilis* inulase.
(a) Inulin (9.00 min)
(b) Glucose (15.39 min)
(c) Fructose (22.30 min)

assay condition except for using 100 mM substrates were 100, 55, and 22, respectively. It could also hydrolyze bacterial levan (from *Aerobacter levanicum*) with low affinity. Although it could hydrolyze levan, it is not certain whether the enzyme can split off fructose units coupled together through β-2,6-bonds in the levan chain as well as β-2,1-bound fructose units on branching points. The enzyme has no action on melezitose (3-O-α-D-glucopyranosyl-β-D-fructofuranosyl-α-D-glucopyranoside), suggesting that only a terminal fructosyl unit can be attacked by this enzyme. Under the standard reaction condition, the S/I ratio (the sucrose hydrolyzing activity versus the inulin hydrolyzing activity) appears to be 2.8.

Table 2. Comparison of β -fructofuranosidases isolated from several sources

	Bacillus subtilis	*Kluyveromyces** *fragilis*	*Aspergillus*** *niger*	*Saccharomyces**** spp.
Molecular weight	49,000	–	59,000	270,000
pH optimum	6.6	4.5	5.0	3.5-5.5
Temperature optimum	50°C	50°C	55°C	–
S/I ratio	2.8	26	1.25	1,600*****
K_m for sucrose	10 mM	13.6 mM	–	26 mM

*Workman *et al.* (5)
**Nakamura *et al.* (20)
***Gascon *et al.* (21)
*****GrootWassink *et al.* (22)

Discussion

Previous studies of inulase have been done almost entirely with enzymes of yeast origin. While there are a number of reports of some type of β-fructofuranosidase being produced by several yeast and fungi species, bacterial inulase has been rarely purified and characterized (12, 13). Among them, the bacterial inulase from *Althrobacter ureafaciens* converts inulin to α -D-fructo-furanose β-D-fructofuranose 2', 1:2,3'-dianhydride and small quantities of other oligofructose units (19). Therefore, it is not adequate as an enzyme source for the production of fructose. Recently, we found that a strain of *B. subtilis* could grow rapidly in inulin containing media. This finding suggest that *B. subtilis* can be used as the source of inulase. Generally, *B. subtilis* is one of the bacteria that produce commercially important enzymes such as α -amylase, proteases, and penicillinase. In addition, significant advances have been made in understanding the details of *Bacilli* genetics in recent years. As a potential inulase source, we have tried to investigate the properties of inulase from *B. subtilis*.

It is known that many microbial inulases from yeasts and fungi catalyze the hydrolysis of sugar posessing a terminal unsubstituted β-D-fructofuranosyl residues. Likewise, *B. subtilis* inulase acts on straight chain oligo- and polyfructosides of the inulin series, as well as on sucrose with high efficiency. Therefore, *B. subtilis* inulase should be classified as β-fructo-furanoside fructohydrolase (EC 3.2.1.26) with a broad substrate spectrum. Other microbial inulases having a different mode of action have been reported. *A. niger* Type III inulase purified by Nakamura *et al.* (9) had no activity towards sucrose and formed mainly inulotrioses, -tetraoses, and -pentoses from inulin. Therefore, this endo acting inulase is separately classified as β -2,1-fructanohydrolase (EC 3.2.1.7).

A comparison of the enzyme from *B. subtilis* with those from other microorganisms (Table 2) reveals that some properties are shared. They include similar ranges of substrate specificities, higher affinity for sucrose than inulin, and almost the same temperature optima. The pH for optimal activity of *B. substilis* inulase is in the nearly neutral region similar to that of the *Arthrobacter ureafaciens* enzyme. In contrast, fungal and yeast inulases exhibit acid pH optima, i.e., an optimum between pH 4.5 and 5.0 for inulase from *K. fragilis* and *A. niger* (3, 9). *Arthrobacter ureafaciens* inulase showed a pH optimum of 6.0 and was rather stable over the pH range from 4.0 to 11.0 (12). However, *B. subtilis* inulase lost its activity almost entirely after incubation of 1 hr at pH 5.0. This made difficulties in performing the further purification of the enzyme by preparative isoelectric focusing. Although we have tried to purify the enzyme to homogeneity, our trial is thus far unsuccessful due to the low activity yield. Further attempts to improve purity are now under consideration.

요 약

Bacillus subtilis 의 세포내 이눌라아제가 부분정제되고 그의 작용 모드와 일반적 특성이 조사되었다. 이 효소는 gel filtration에 의하여 분자량을 추정하였을 때 49,000이었고, 등전점은 5.2이었다. 기질에 대한 친화성의 지표인 K_m값은 설탕에 대해서는 10mM, 라피노오스에 대해서는 18mM이었다. 이 효소는 산성쪽에서는

불안정한 단백질로서 pH6.6에서 최대 활성을 보였으며 최적온도는 10분간 반응시켰을 때 50℃였다. 이 효소의 작용모드는 이눌린같은 구조를 가지는 과당 중합체를 과당 끝부분으로부터 하나씩 잘라가는 exo-cleavage 형이었다.

References

1. Windholz, M.: The Merck Index, Merck & Co., Rahway, N.J., p.725 (1983).
2. Fleming, S.E. and J.W.D. GrootWassink: *CRC Crit. Rev. Food Sci. Nutr.* **12,** 1 (1979).
3. Synder, H.E. and H.J. Phaff: *Antonie van Leeuwenhoek,* **26,** 433 (1960).
4. Nahm, B.H. and S.M. Byun: *Korean Biochem. J.* **10,** 95 (1977).
5. Workman, W.E. and D.F. Day: *FEBS Lett.* **160,** 16 (1983).
6. Negoro, H. and E. Kito: *J. Ferm. Tech.* **51,** 96 (1973).
7. Beluche, I., J.P. Guiraud and P. Galzy: *Folia Microbial.* **25,** 32 (1980).
8. Demeulle, S., J.P. Guiraud and P. Galzy: *Z. Allg. Microbiol.* **21,** 181 (1981).
9. Nakamura, T. and T. Kurokawa: *Nippon Nogei Kagakukaishi,* **52,** 159 (1978).
10. Zittan, L.: *Starch,* **33,** 373 (1981).
11. Nakamura, T. and S. Nakatsu: *Nippon Nogei Kagakukaishi,* **51,** 681 (1977).
12. Uchiyama, T., S. Niwa and T. Tanaka: *Biochim. Biophys. Acta.* **315,** 412 (1973).
13. Chung, K.Y., K.H. Park and K.H. Lee: *Korean J. Food Sci. Technol.* **13,** 67 (1981).
14. Uhm, T.B., J.S. Hong, H.S. Sohn, M.K. Park and S.M. Byun: *J. Korean Agr. Chem. Soc.* **28,** 131 (1985).
15. Miller, G.L.: *Anal. Chem.* **31,** 426 (1959).
16. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall: *J. Biol. Chem.* **193,** 265 (1951).
17. Andrews, P.: *Biochem. J.* **96,** 595 (1965).
18. Hanes, C.S.: *Biochem. J.* **26,** 1406 (1932).
19. Uchiyama, T.: *Biochim. Biphys. Acta.* **397,** 153 (1975).
20. Nakamura, T, S. Maruki, S. Nakatsu and S. Ueda: *Nippon Nogei Kagakukaishi,* **52,** 581 (1978).
21. Gascon, S., N.P. Neumann and J.O. Lampen: *J. Biol. Chem.* **243,** 1573 (1968).
22. GrootWassink, J.W.D. and S.E. Fleming: *Enzyme Microb. Technol.* **2,** 45 (1980).

(Received July 18, 1990)