Korean J. Food & Nutr. Vol. 14. No. 3, 193~198 (2001)

THE KOREAN JOURNAL OF
FOOD AND NUTRITION
한국식품영양학회지

Immobilization and Characterization of Inulinase from *Bacillus sphaericus* 188-1

Na-Mi Kim*, Young-Geun Ann and Jong-Soo Lee†**

Korea Ginseng & Tabacco Research Institute, Taejon 305-345, Korea
Faculty of Food Science, Chungcheong College, Cheongwon 363-792, Korea
Dept. of Genetic Engineering, Paichai University, Taejon 302-735, Korea

Bacillus sphaericus 188-1이 생성하는 Inulinase의 고정화와 특성

김나미* · 안용근 · 이종수†**

배재대학교 유전공학과, *한국인삼연초연구원, **충청대학 식품과학부

Abstract

Inulinase from *Bacillus sphaericus* 188-1 was partially purified and immobilized on oxidized cellulose for continous production of fructose syrup. The immobilized enzyme had 47 unit of inulinase activity per g of oxidized cellulose, with the immobilization yield of 41% and the activity yield of 39%. Optimal reaction temperature and pH of the immobilized inulinase on inulin were 50℃ and pH 9.0, respectively, and it was stable up to 50℃ and pH 8.0~10.0. Activity of the immobilized inulinase was activated by K^+, Ca^{2+}, $Mn^{2+}$and Hg^{2+}, but significantly inhibited by EDTA(10mM).

Key words : immobilization, characterization, inulinase, *Bacillus sphaericus* 188-1.

INTRODUCTION

Inulinase (β-fructosidase : β-fructan hydrolase : 2,1-D-fructan-fructanohydrolase ; EC 3.2.1.7) splits β-2,1-fructanofuranosidic linkages of inulin to produce fructose[1]. The classification of inulinase was based on the occurrence of inulinase, activity on sucrose and inulin, type of reaction products (oligofructans or only fructose), secretion pattern (exo or endo) etc.

These inulinase split fructans of the inulin type either endo- or exo-wise, producing a series of oligofructans or fructose but rarely hydrolyze sucrose.

Inulinase from yeasts, in particular, is able to hydrolyze inulin and levan-type fructans exo-wise and is an extracellular enzyme partially associated with the cell wall and partially excreted into the culture fluid[2]. Almost of inulinases were glycoproteins which can be induced exclusively by inulin[1].

The inulinase has been found in several yeasts and bacteria such as *Kluyveromyces* sp.[3~6], *Debaryomyces* sp.[7], *Saccharomyces* sp.[8,9], *Streptococcus* sp.[10,11], *Clostridium* sp.[12], *Arthrobacter* sp.[13,14], *Chrysosporium* sp.[15], and in some mold such as *Aspergillus* sp.[16~18], *Penicillium* sp.[19] and *Fusarium* sp.[20]. However, only inulinases from some yeast strains have exclusively been used in food and pharmaceutical industries. Generally, immobilized inulinase was used in the continuous production of fructose from inulin[21~23]. A preference for some

† Corresponding author : Jong-Soo Lee

specific methods and immobilizing agents (support matrix) is widely used in the immobilization, for example : adsorption and cross linking with glutaraldehyde, covalent coupling with porous silica or cellulose derivatives, etc. An interesting method is entrapment in fibers of polymers, which have been successfully developed and applied in the commercial and industrial scale. However, the support matrix is very expensive[24].

In order to select inexpensive support matrix for immobilization of the inulinase from *Bacillus sphaericus* 188-1 and use in the mass production of fructose from inulin, we immobilized the inulinase on various support matrix and characterized.

MATERIALS AND METHODS

1. Strains and culture condition

Bacillus sphaericus 188-1 isolated from soil was used as a source of inulinase[25]. It was cultivated in inulin broth(0.5% inulin, 0.4% Bacto-peptone, 10mM $MgSO_4 \cdot 7H_2O$ (pH 7.5)).

2. Assay of inulinase activity

Inulinase activity was measured by determining the amount of the released reducing sugar from inulin[26]. The reaction mixture containing 0.8 ml of 1% inulin in sodium phosphate buffer (pH 7.0) and 0.2 mL enzyme solution was incubated at 37℃ for 1 hr. Total reducing sugar was measured by the Somogyi- Nelson method[27]. D-Glucose was assayed using the Glucose [HK] kit. D-fructose was determined as difference between the amount of total reducing sugar and D-glucose. One unit of inulinase activity is defined as the production of 1 μM of the product per minute at 37℃ and specific activity was expressed as unit per mg protein.

3. Partial purification and immobilization of the inulinase

Intracellular inulinase from *Bacillus sphaericus* 188-1 was partially purified by $(NH_4)_2SO_4$ precipitation (30~70% saturation), followed by ion-exchange chromatography on DEAE-Sephadex A-50.

The inulinase was immobilized by the methods of schiff base immobilization as follows. Schiff base immobilization of the inulinase by the cross-linkage method was based on the formation of a Schiff base between a free aldehyde groups of support (oxidized cellulose by $NaIO_4$) and lysine residue in amino group of the enzyme.

For the preparation of the support materials (oxidized cellulose derivatives and oxidized defatted cotton) 2% suspension of cellulose and defatted cotton in 0.2 M sodium metaperiodate was incubated at 4℃ for 5 hrs, and washed by distilled water and then lyophilized.

According to Ann's method[28~30], portion (50 mg) of the partial purified inulinase from *Bacillus sphaericus* 188-1 was reacted with 250 mg of the oxidized cellulose derivatives and defatted cotton in 10 ml of 50 mM sodium phosphate buffer (pH 6.5) at 37℃ for 30 min. The immobilized enzyme was suspended in 10 ml of sodium phosphate buffer (pH 6.5) and stored at 4℃ for further experiments.

The immobilization yield (%) was determined by (amount of immobilized protein / amount of protein loaded) × 100. The activity yield of the immobilized enzyme (%) was determined by (inulinase activity of the immobilization enzyme / inulinase activity loaded) × 100.

RESULTS AND DISCUSSION

1. Partial purification and immobilization of inulinase

The partial purified enzyme showed 121 unit/mL of inulinase activity and 50 unit/mg protein of the specific activity. The partial purified inulinase was immobilized on various support materials and determined its immobilization yield and activity yield (Table 1).

When inulinase of *B. sphaericus* 188-1 was immobilized on 5 hrs-oxidized cellulose, its immobilization yield(41.0%) and activity yield(39.0%) were maximized. Therefore, we finally selected 5 hrs- oxidized cellulose as support matrix for the inulinase immobilization.

Table 1. Immobilization of inulinase from *Bacillus sphaericus* 188-1 onto oxidized cellulose and defatted cotton

Support materials	Oxidation time by $NaIO_4$	Activity when loaded		Activity in immobilization (U/g wet wt. of materials)	Total supernatant protein (mg)	Immobilization yield (%)[b]	Activity yield (%)[b]
		Total protein (mg)	Activity[a] (U/ml)				
Cellulose	40 min	2.4	121	7.02	1.87	22	5.8
	5 hr	2.4	121	47.33	1.41	41	39
Defatted cotton	5 hr	2.4	121	17.3	1.58	34	14.3

[a] One unit of the activity was defined as the amount of enzyme that required to produce reducing sugar equivalent to 1 μM of glucose per min.

[b] See Materials and Methods for definition.

2. Properties of the immobilized inulinase

The effects of temperature on the activity of the immobilized inulinase was given in Fig. 1. Optimal reaction temperature was 50℃, which was higher than those of the original enzyme(40℃) and garlic inulinase (40℃)[31].

The thermal stability of the immobilized enzyme was investigated and compared with that of the original enzyme. As shown in Fig. 2, the immobilized enzyme was relatively stable, showing 50% residual activity at 50℃ for 20 min wherease original enzyme had 0% residual activity at 50℃ for 20 min[31]. These results suggest that inulinase immobilization onto oxidized cellulose is very effective in increasing of

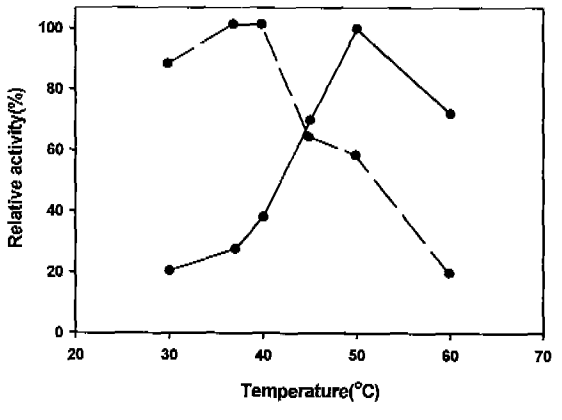

Fig. 1. Optimal reaction temperature of the native inulinase and the immobilized inulinase onto oxidized cellulose. Native inulinase : ---, Immobilized inulinase : ——.

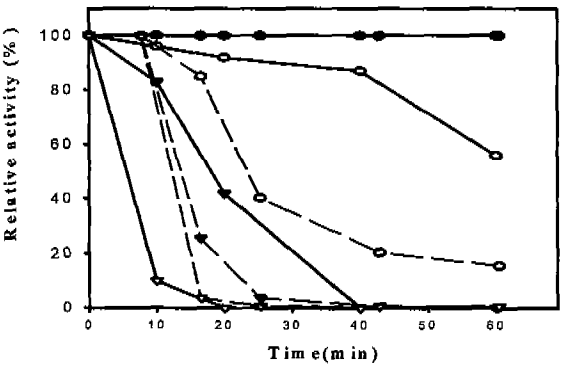

Fig. 2. Thermal stability of the native inulinase and the immobilized inulinase onto oxidized cellulose. ● : 30℃, ○ : 40℃, ▼ : 50 ℃, ▽: 60℃ Native inulinase : ---, Immobilized inulinase : ——.

their thermal stability and such increase of thermal stability appear to be important for industrial application of inulinase.

Optimal reaction pH of the immobilized enzyme was 9.0 and it was stable at the range of pH 8.0~10.0. These results were different with that of original enzyme (optimum pH : 6.5, stable at pH 6.0 ~8.0)[31] (Fig. 3).

The effects of metal ions and EDTA on the activity of the immobilized inulinase was shown in Table 2. The activity of the immobilized inulinase was activated by K^+, Ca^{2+}, $Mn^{2+}$and Hg^{2+}, and its activity was also higher than that of original enzyme[31].

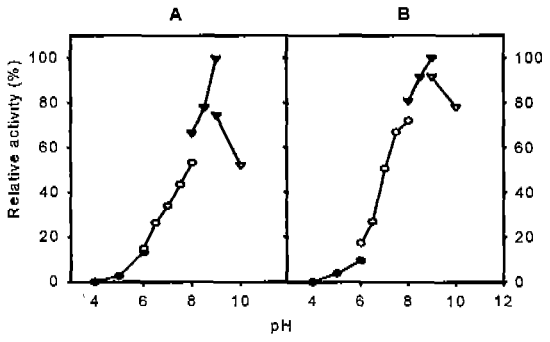

Fig. 3. Optimal pH(A) and pH stability(B) of the immobilized inulinase onto oxidized cellulose. ● : Citrate buffer (pH 4.0~6.0), ○ : Phosphate buffer (6.0~8.0), ▼: Tris buffer (pH 8.0~9.0), ▽: Borate buffer (pH 9.0~10.0) (Opt. pH and pH stability of the native inulinase : pH 6.5, pH 6.0~8.0)[31].

All of above results strongly suggest the oxidized cellulose may be used industrially for conversion of unstable inulinase against thermal or metal ions to stable inulinase. Some properties of the immobilized enzyme was compared with those of the original enzyme[31] in Table 3.

3. Operation stability of the immobilized inulinase

The operational stability of the immobilized inulinase was studied during a prolonged period for complete hydrolysis of inulin : the continuous hydrolysis of a 1% inulin solution at a flow rate of 3mL/hr was performed at 40℃ by measuring the reducing sugars released in the effluent (Fig. 4). Complete hydrolysis of inulin to reducing sugars continued for 6 hrs at 40℃ without any microbial contamination of packed bed column. The operation stability of the immo-

Table 2. Effects of metal ions and EDTA on the activity of native inulinase and the immobilized inulinase onto oxidized cellulose

Reagents*	Relative activity (%)	
	Native inulinase	Immobiolizes inulinase
Control	100	100
K^+	101	143
Na^+	100	76
Li^+	98	85
F^+	103	103
Cu^{2+}	3	97
Ca^{2+}	89	134
Fe^{2+}	52	81
Mg^{2+}	88	107
Mn^{2+}	78	111
Zn^{2+}	-	93
Cd^{2+}	25	81
Hg^{2+}	10	115
Al^{3+}	87	68
EDTA (1mM)	95	78
(10mM)	74	14

*All of the cations were added as chlorides, except Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} and Al^{3+}, which were added as sulfates. The enzyme solutions containing 1 mM metal ions and EDTA except 25 mM of K^+ and Na^+ were preincubated at 37℃ for 2 hr, respectively and the relative activity was measured.

Table 3. Comparison with some properties between the original inulinase and immobilized inulinase

Properties	Original enzyme	Immobilized enzyme
Specific activity(U/mg protein)	3700	121
Optimal reaction temperature	40℃	50℃
Thermal stability	20 % RA[1] at 40℃ for 20 min	50% RA at 50℃ for 20min
Optimal reaction pH	pH 6.5	pH 9.0
pH stability	pH 6.0~8.0	pH 8.0~10.0
Inhibitor(metal ions)	Cu^{2+}, Cd^{2+}, Hg^{2+}	only 10mM EDTA

[1] RA means residual activity.

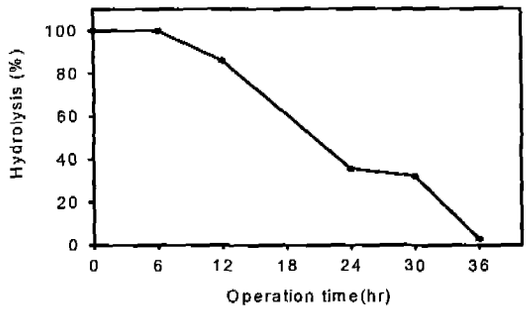

Fig. 4. Operational stability of the immobilized inulinase onto oxidized cellulose. 1 % inulin solution (pH 7.5) was hydrolyzed continuously with a flow rate of ml/hr at 40℃.

bilized inuilnase was lower than those of immobilized inulinase with aminocellulofine[24], with open pore gelatin matrix[23] and with aminoethylcellulose[21].

요 약

고정화 inulinase를 이용하여 inulin으로부터 fructose시럽을 연속적으로 생산하기 위하여 *Bacillus sphaericus* 188-1 이 생성하는 inulinase를 부분 정제한 후 5시간 $NaIO_4$로 산화시킨 cellulose에 고정화시킨 다음 이들의 성질을 조사하였다. 산화 cellulose에 고정화시킨 inulinase의 활성은 g당 47 Unit 이었고 고정화수율과 활성수율은 각각 41%와 39% 이었다. 고정화시킨 inulinase의 작용 최적온도와 pH는 각각 50℃, pH 9.0이었고 50℃, pH 8.0~10.0에서 비교적 안정 하였다. 고정화 시킨 inulinase의 활성은 K^+, Ca^{2+}, Mn^{2+}과 Hg^{2+}에 의하여 활성화 되었으며 EDTA (10mM)에 의하여 심하게 저해되었다.

REFERENCES

1. Vandamme. E. J. and Derycke, D. G. : Microbial inulinase fermentation process, properties and application. *Adv. Appl. Microbiol.*, 29, 193 (1983).
2. Rouwenhorst, R. J., Visser, L. E., van der Baan, A. A., Sheffers, W. A. and van Dijken, J. P. : Production, distribution and kinetic properties of inulinase in continuous cultrues of *Kluyveromyces marxianus* CBS 6556. *Appl, Environ. Microbiol.*, 54, 1131~1137 (1988).
3. Negore, H. : Inulase from *Kluyveromyces fragilis*. *J. Ferment. Technol.*, 56, 102~107 (1978).
4. Parekh, S. and Margaritis, A. : Production of inulinase (β-fructanhydrolase) by *Kluyveromyces marxianus*. *Agric. Biol Chem.*, 50, 1085~ 1091 (1986).
5. Rouwenhorst, R. J., Ritmeester, W. S., Scheffers, W. A. and van Dijken, J. P. : Localization of inulinase and invertase in *Kluyveromyces* species. *Appl. Environ. Microbiol.*, 56, 3329~3335 (1990).
6. Laloux, O., Cassart, J. P., Delcour, J., van Beeumen, J. and Vandenhaute, J. : Cloning and sequencing of the inulinase gene of *Kluyveromyces marxanus* var. *marxianus* ATCC 12424. *FEBS Lett.*, 289, 64~69 (1991).
7. Beluche, J., Guiraud, J. P. and Galzy, P. : Inulinase activity of *Debaromyces cantarellii*. *Folia microbiologica*, 25, 3~8 (1980).
8. Snyder, H. E., and Phaff, H. J. : Studies on a beta-fructosidase(inulinase) produced by *Saccharomyces fragilis*. Antonie van Leeuwenhoek *J. Microbiol. Serol.*, 26, 433~439 (1960).
9. Snyder, H. E. and Phaff, H. J., : The pattern of action of inulinase from *Saccharomyces fragilis* on inulin. *J. Biol. Chem.*, 237, 2483~2488 (1962).
10. Burne, R. A., Schilling, K., Bowen, W. and Yasbin, R. E. : Expression, purification and characterization of an exo-β-D-fructosidase of *Streptococcus mutans*. *J. Bacteriol.*, 169, 4507~4511 (1987).
11. Takahashi, N., Mizuno, F. and Takamori, K. : Purification and preliminary characterization of exo-β-D-fructosidase in *Streptococcus salivarius* KTA-19. *Infect. Immun.*, 47, 271~276 (1985).
12. Efstathiou, I., Reysset, G. and Truffaut, N. A. : Study of inulinase activity in the *Clostridium acetobutylicum* strain ABKn8. *Appl. Microbiol. Biotechnol.*, 25, 143~148 (1986).
13. Uchiyama, T., Niwa, S. and Tanaka, K. : Purification and properties of *Arthrobacter ureafaciens* inulinase II. *Biochem. Biophys. Acta*, 315, 412~ 417 (1973).
14. Haragushi, K., Hayashi, K. and Kasumi, T. : Purification and properties of inulinase from *Arthrobacter globiformis*. *Starch*, 42, 28~35 (1990).
15. Xiao, R., Tanida, M. and Takao, S. : Purification and characteristics of two exoinulinase from *Chrysosporium pannorum*. *J. Ferment. Bioeng.*, 67, 331~336 (1989).
16. Nakamura, T., Kurokawa, T., Nakatsu, S. and Ueda, S. : Crystallization and general properties of an extracellular inulinase from *Aspergillus* sp. *Nippon Nogei. Kagakukoishi*, 52, 159~164 (1978).
17. Derycke, D. G. and Vandamme, E. J. : Production and properties of *Aspergillus niger* inulinase. *J. Chem. Technol. Biotechnol.*, 34, 45~50 (1984).

18. Ettalibi, M. and Baratti, J. C. : Molecular and kinetic properties of *Aspergillus ficuum* inulinase. *Agric. Biol. Chem.*, 54, 61~66 (1990).
19. Nakamura, T. and Nakatsu, S. : Genertal properties of extracellular inulase from *Penicillium*. *Nippon Nogeikagaku Kaishi*, 51, 681~686 (1977).
20. Gupta, A. K., Rathore, P., Kaur, N. and Singh, R. : Production, thermal stability and immobilization of inulinase from *Fusarium oxysporum*. *J. Chem. Technol. Biotechnol.*, 47, 245~251 (1990).
21. Kim, W. Y. and Byun, S. M. : Hydrolysis of inulin from *Jerusalem artichoke* by inulinase immobilized on aminoethylcellulose. *Enzyme Bicrob. Technol.*, 4, 239~244 (1982).
22. Kim, C. H. and Rhee, S. K. : Fructose production from *Jerusalem artichoke* by inulinase immobilized on chitin. *Biotechnol. Lett.*, 11, 201~207 (1989).
23. Bajpai, P. and Margarites, A. : Immobilization of *Kluyveromyces marxianus* cells containing inulinase activity in open pore gelatin matrix. 2. Application for high fructose syrup production. *Enzyme Microb. Technol.*, 7, 459~465 (1985).
24. Nakamura, T., Ogata, Y., Shitara, A., Nakamura, A and Ohta, K. : Continuous production of fructose syrups from inulin by immobilized inulinase from *A. niger* mutant 817. *J. Ferment. Bioeng.*, 80, 164~169 (1995).
25. Kwon, S. J., Yoo, J. Y. and Lee, J. S. : Isolation of intracellular inulinase-producing bacterium and culture condition for inulinase production. *Food Sci. Biotechnol.* 8, 24~29 (1999).
26. Lee, T. K., Sung, H. C., Choi, Y. J. and Yang, H. C. : Culture condition for inulinase production by *Pseudomonas* species. *Kor. J. Appl. Mocrobiol. Bioeng.* 15, 176~181 (1987).
27. Chaplin, M. F. and Kennedy, J. F. : Cabohydrate analysis :Practical approach. IRL Press, Oxford. pp. 3~5 (1986).
28. Ann, Y. G., Iizuka, M, Minamiura N. and Yamamoto, T : Evidence for he existence of an active monomer of sweet potato β-amylase. *Agric. Biol. Chem.*, 53. 3109~3110 (1989).
29. Ann, Y. G., Iizuka, M., Yamamoto, T. and Minamiura, N. : Preparation and some properties of active monomer of sweet potato β-amylase. *Agric. Biol. Chem.*, 54(3), 769~774 (1990).
30. Ann, Y. G., Iizuka, M, Minamiura N. and Yamamoto, T. : Active monomer of sweet potato β-amylase: Stabilization and an improved preparation method using α-cyclodextrin. *J. Fermant. Bioeng.*, 70(2), 75~79 (1990).
31. Kwon, S. J. : Biochemical and biotechnological aspects of intracellular invertase and inulinase, Ms thesis of Paichai Univ. (1998).

(2001년 4월 10일 접수)