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A Constitutive Inulase from a *Bacillus*

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Bacillus 균이 생산하는 구성효소로서의 이눌라아제

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초 록

지금까지 보고된 바와는 다른 이눌라아제를 체외로 분비하는 한 세균을 분리하였고 이 균은 이눌린이 존재하지 않는 설탕, 세룰로오스, 포도당이 포함된 배지에서 이눌라아제를 생산하는 구성 효소로서의 특성을 나타내었다. 또한 동일한 배지 조건에서 강력한 이눌라아제를 생산하는 것으로 알려진 *Kluyveromyces fragilis*와 유사한 효소 활성을 짧은 배양 시간내에 나타내었다. Bergey's manual에 따라 조사된 이 균의 특징은 호기성, 그람 양성, 간균으로서 포자를 형성하여 *Bacillus subtilis*로 추정되었다.

Introduction

Inulin, a reserve carbohydrate in plants of Jerusalem artichoke, chicory, and dahlia, 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 prefered because of a lower byproduct formation than that by acids[2]. The enzyme inulase, which splits β-2, 1-fructofuranosidic bonds of inulin, is an extracellular glycoprotein produced by a number of yeasts and filamentous fungi. Inulases had been described mainly from yeasts such as *Kluyveromyces*[3~5], *Candida*[6] and *Debaryomyces* species[7~8], but also fungal inulases from *Aspergillus*[9~11] and *Penicillium* species[12] were reported. Generally, *Kluyveromyces fragilis* had been most extensively studied due to the high yields of inulase production[6,13]. However one limitation in the utilization of the enzyme is that the most inulases reported so far from various microorganisms were the inducible en-

zymes, which require inulin as a carbon source in the media.

Recently, we isolated a bacterium which has never been described as an inulin hydrolyzing microorganism previously and this bacterium produced relatively higher activity of inulase in the media containing glucose without inulin as carbon sources.

This paper describes a constitutive inulase of microorganism isolated from decayed artichoke tubers, as well as physiological properties of this microorganism identified as *Bacillus subtilis* tentatively.

Materials and Methods

Materials

Jerusalem artichoke tubers, harvested in late fall from a farm near Chonju, Korea were stored at −4°C until used. Tubers were peeled and cut into slices. The slices were then freeze-dried and packed in laminated polyethylene packs. Authentic inulin was purchased from Sigma Chemicals(St. Louis, U.S.A.). All other chemicals used were commerically available reagent grades. *Kluyveromyces fragilis* strain No. 351 was used as the source of inulase, which was supplied by Snyder and Phaff, University of California, Davis.

Isolation and identification of microorganism

All tests for isolation and identification of inulin hydrolyzing microorganisms were carried out according to the Manual of Methods for General Bacteriology, ASM[14] and the Bergey's Manual of Determinative Bacteriology[15]. As a comparative purpose, authentic *Bacillus subtilis* ATCC 14593 was also used for the tests. The microorganisms for the tests were collected from soil of various districts, air and decayed artichoke tubers. The organisms collected were cultivated on agar plate containing media described in Table 1. By a serial dilution method single colonies were obtained.

Culture conditions

Single colonies isolated were used as the source of inulase. Cutlures were maintained on 3% artichoke tuber extract-agar slants. The culture medium composition was one as described in Table 1 with an initial pH of 6.8. For the production of inulase, cells were grown with vigorous shaking(150rpm, amplitute 50mm) in 250m*l* of Erlenmeyer flasks which were filled to 1/10 volume with the media. After the culture for 8 h at 37°C, cells were harvested by centrifugation at 5,000g for 20min. The supernatant which has extracellular inulase was used as crude enzyme preparation.

Enzyme Assay

Inulase activity was measured by determining the released reducing sugar from inulin by the 3,5-dinitrosalicylic acid method[16]. The incubation mixture contained 1m*l* of 5% inulin in 0.1 M sodium acetate buffer, pH 5.0 and 0.2m*l* of the crude enzyme solution. After incubation for 10min at 30°C, the reaction was stopped by adding 1 m*l* of 3,5-dinitrosalicylic acid reagent. One unit of inulase activity was defined as one micromole of fructose produced per minute at 30°C.

Protein Assay

The concentration of protein was determined according to the method of Lowry et al[17] using bovine serum albumin as a standard protein.

Results and Discussion

Identification

Inulin hydrolyzing microorganisms were collected from soil, air and decayed artichoke tubers. The isolated colonies were cultivated on agar plates containing media described in Table 1. Several microorganisms including yeasts and fungi were observed. Among 65 yeasts, 42 fungi and 78 bacteria isolated, microorganism which had higher inulase activities than those of *K. fragilis* were selected. As shown in Fig.

Table 1. Media composition*(%)

Jerusalem artichoke tuber(dried)	3
Yeast extract	0.3
$(NH_4)_2HPO_4$	1
$FeSO_4$	0.015
$MgSO_4 \cdot 7H_2O$	0.05
KCl	1.5

*For the agar plate 1.5g of agar (1.5%) were added to above media.

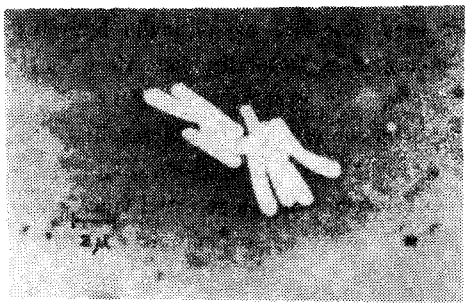

Fig. 1. Negative staining of the inulin hydrolyzing microorganism isolated.

1, one bacterium revealed particularly very high inulase activity. Therefore this bacterium was characterized. The isolated microorganism had bacterial sizes and showed aerobic, rod typed, spore forming and Gram positive bacterium. According to the *Bergey's manual*, the bacterium having these properties was classified as *Bacillus* species. Further classification were performed as summarized in Table 2. Considering the morphological and biochemical data and the properties of *Bacillus subtilis* ATCC 14593, the bacterium appears to be *Bacillus*

Table 2. Identification of the inulin hydrolyzing microorganism

Identification method	Characters of IHM	Remark
A. Morphological properties		
1. Type & Size	Rod, seldom in chain 2~3μm×0.5~0.7μm	
2. Gram staining	Positive	
3. Colonies on agar media	Irregular, surface dull wrinkled, active spreading	
4. Appearance in broth culture	Dull, wrinkled, coherent pellicle	
5. Motility	Active motility	
6. Flagella	+	Schaeffer-Fulton method
7. Spore	Endospore forming, elliptical, central position	
B. Biochemical properties		
1. Products of action on glucose, arabinose, xylose and mannitol		
1. Gas	No gas	
2. Acid	All +	
2. Gelatin hydrolysis	Liquefied to one or more centimeter (20°C, 5 days)	
3. Catalase test	+	
4. Egg york reaction	—	
5. Growth in 7% NaCl & in sabourod dextrose agar	+	
6. Citrate-salt agar	Alkali reaction	Simmons citrate agar
7. Casein & starch hydrolysis	All +	
8. Nitrate reduction	+	
9. Litmus	Reduction	

subtilis. Until recently, *Bacillus sabtilis* has not been described as an inulin hydrolyzing microorganism. Generally, bacteria have more advantages in enzyme production than yeasts and fungi due to their rapid metabolic turnover rate. In these respects, *Bacillus*, rapidly growing bacteria, will be a potential inulase source if culture conditions are optimized.

Enzyme induction

The growth of cells and the change of enzyme activity during culture were shown in Fig. 2.

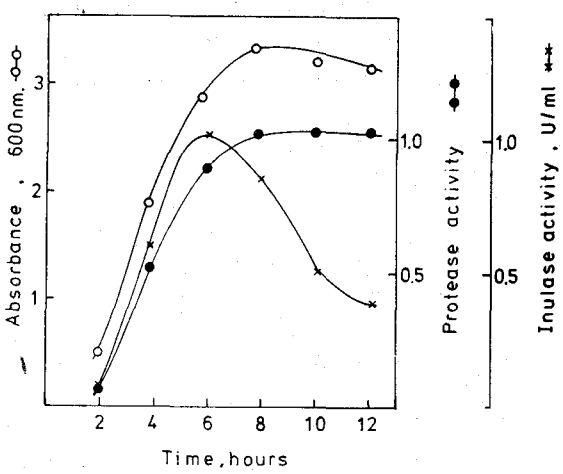

Fig. 2. Growth and inulase and proteses activities during the culture of a *Bacillus* isolated.

Interestingly, the results showed that inulasewas induced during log phase and sharply decreased at stationary phase. Although this phenomenon was not explained exactly, it seemed due to the high protease activities in the media. From preliminary experiments, protease activities were increased to the maximal level at the stationary phase. Therefore, it assume that the protease may hydrolyze the inulase presented media. The effect of various carbon sources in the media on the inulase production was investigated. As the result, the bacterium produced relatively higher activity of inulase in media containing glucose without inulin material as a carbon source(Table 3). This result showed that the *Bacillus* can produce extracellular inulase constitutively. Also, compared to the inulase activity of *K. fragilis*, the bacterium thought to be a promising inulase producer. The *de novo* biosynthesis of certain enzymes of a microbial cell involved in catabolism of disaccharides, for example, are subject to a switch-on mechanism elicited by a corresponding component of the surrounding medium. Invertase and inulase behave differently with respect to their induction. The literature contains numerous more or less well supported observations that invertase of *Saccharomyces cerevisiae* is a constitutive enzyme, although its concentration can be increased by sucrose in the medium. Most inulase reported, however, are inducible enzymes and its formation is stimulated best by inulin. Since yeast cell membranes are impermeable to fructosides, including sucrose[18], the primary physiological function of inulase dictates an external location. Recently, it showed that fructose was identified as the primary physiological inducer during carbon limited continuous culture of *K. fragilis*[19]. Therefore the constitutive formation of inulase from *Bacillus subtilis* can be explained by several considerations such as the mutation of control gene, insertion of phage DNA fragment having inulase gene, and certain inducer effects. Howe-

Table 3. Inulase activity during the culture in different carbon sources (unit/m*l* crude supernatant)

Organism \ Carbon source	Sucrose	Cellulose	Glucose	Inulin	Artichoke tuber
B. subtilis	0.6	0.1	0.8	0.7	1.1
K. fragilis	0.4	0	0	0.6	0.9

Table 4. Inulase production of a *Bacillus* during the culture in various media (unit/m*l* crude supernatant)

Medium \ Time	6 h	12 h	18 h
**Bacillus subtilis* medium 1	0.9	0.7	0.5
***K. fragilis* medium	0.9	0.4	0.2
****Bacillus subtilis* medium 2	0	0	0

*Glucose 30g, ammonium phosphate, dibasic 7g, KH_2PO_4 1.5g, $MnCl_2 \cdot 4H_2O$ 40mg, $MgSO_4 \cdot 7H_2O$ 0.5g, $CaCl_2 \cdot 2H_2O$ 0.3g, $FeSO_4 \cdot 7H_2O$ 2.5mg, and ammonium molybdate 2mg per liter. pH not adjusted.

**Described in Table 1.

***Sodium chloride 5 g and nutrient broth 8 g per liter. pH not adjusted.

ver, the constitutive formation of inulase should be investigated in detail in the molecular level.

Inulase activities during the culture in several *Bacillus* media were determined(Table 4). In medium containing no carbon source, the organism did not excrete the enzyme at all. The inulase activities in the *Bacillus subtilis* medium 1 and the *K. fragilis* medium were similar level at the culture of 6 h. However, inulase activities in the *K. fragilis* medium were dropped to the lower level than those in the *Bacillus* medium 1 after the culture of 6 h. This result can be explained by the synthesis level of proteases as stated earlier. That is, it assumes that bacteria growing in the rich nitrogen compounds stimulate the synthesis of proteases for their rapid metabolic turnover rate.

Recently, many microorganisms have been isolated and utilized for the production of fructose. In the future, direct production of fructose from Jerusalem artichoke will be considered in the view point of the cost and the commercial requirement of high concentrated fructose syrup. In this respects, a constitutive inulase from a *Bacillus* species will be served as one of the most promising inulase sources if the culture conditions would be well optimized.

Abstract

Recently, we investigated characteristics of a bacterium which has never been reported as an inulin hydrolyzing microorganism. Several properties of the isolated microorganism were aerobic, rod typed, spore forming and Gram positive. According to the *Bergey's manual*, this bacterium tentatively appeared to be *Bacillus subtilis*. This bacterium produced inulase constitutively in the media containing glucose, sucrose or cellulose without inulin as a sole carbon source. Also, inulase activities of the bacterium per unit culture volume showed 1.1 unit/m*l* comparable to 0.9 unit/m*l* of *Kluyveromyces fragilis* with a relatively short culture time.

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