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Functional Oligosaccharide DFA III Forming Enzyme Produced by Microorganisms

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Abstract

Inulin is a polysaccharide found in various plants (chicory, dahlia, and so on). A unique oligosaccharide DFA III (di-fructose di-anhydride III) is formed from inulin with a special microbial enzyme. This enzyme was named as inulin fructotransferase (DFA III-producing) [EC 4.2.2.18]. The oligosaccharide DFA III has sweetness half as sucrose. The DFA III also has a special quality that promotes the assimilation of minerals (Ca, Fe, and so on) from intestines. Therefore, the oligosaccharide DFA III has a unique potential for the prevention of osteoporosis and iron deficiency anaemia. The sales in the market of DFA III were started in 2004 in Japan. Now, we can purchase a product of supplement containing the DFA III and a mineral in a pharmacy and a convenience store in Japan. In this article, we summarize the research on the oligosaccharide DFA III forming enzyme produced by the microorganisms.

Keywords: Inulin; Oligosaccharide DFA III; Enzyme

Introduction

A huge amount of sugar (more than 500,000 tons) is produced from a sugar beet in Hokkaido area, Japan. Therefore, the production of beet sugar is a valuable industry of Hokkaido district, Japan. The consumption of sugar in Japan is gradually decreasing. Accordingly, to introduce an alternative crop for the sugar beet is desirable. In Europe (Germany, Belgium, and so on), a chicory was introduced as an alternative crop for the sugar beet. The root of chicory includes a polysaccharide inulin. The inulin is a storage polysaccharide found in plants (chicory, dahlia, and so on). The inulin is a fructose polymer (β-2,1 linked) terminated by a sucrose residue. In Europe, inulin produced from the chicory is used as a food material. For example, inulin is used as a component of a chocolate (as an alternative material of cocoa butter).

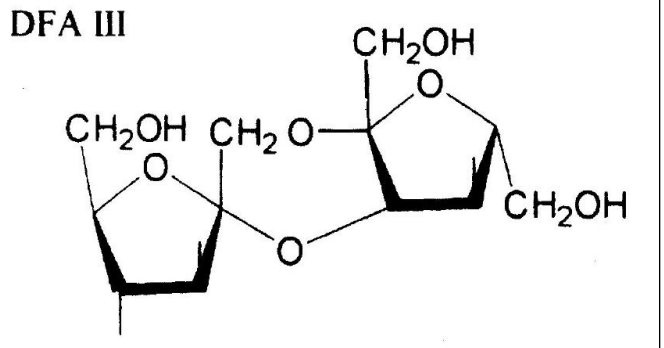

Figure 1: The chemical structure of oligosaccharide DFA III.

On the other hand, by the use of microbial enzymes, all sort of oligosaccharides are formed from inulin. The oligosaccharides have a potential for utilization, as a food, medicine, and so on. We have a unique oligosaccharide DFA III produced from inulin. In the DFA III, two molecules of fructose are bonded to each other at two portions. The DFA III has sweetness half as sucrose. The DFA III is a non-reducing sugar. The chemical structure of the oligosaccharide DFA III is presented in Figure 1. In this article, we summarize the research on the inulin fructotransferase (DFA III-producing) [EC 4.2.2.18] and the functional oligosaccharide DFA III.

Discovery of Oligosaccharide DFA III Forming Enzyme

On the research of inulin decomposing enzymes, inulinases [EC 3.2.1.7] produced by molds and yeasts were studied in the past. Subsequently, new inulin decomposing enzyme was reported by Uchiyama et al., for the first time [1]. This enzyme changed inulin into an oligosaccharide DFA III (di-fructose di-anhydride III) and a residual other oligosaccharides. This enzyme was named as inulin fructotransferase (DFA III-producing) [EC 4.2.2.18]. This new enzyme was produced by a microorganism, *Arthrobacter ureafaciens*. The microorganism *Arthrobacter* is a typical soil bacterium. This new enzyme was produced in a culture supernatant of the microorganism. The enzyme purification was performed with an ammonium sulfate precipitation, an acetone precipitation, and a gel filtration by Sephadex G-100 column. The purity of the enzyme protein was checked by a SDS-PAGE, and a pure enzyme (a single band) was obtained. The maximum activity of the enzyme was observed at pH 6.0 and 50. It was stable up to 50. By a gel filtration, the molecular mass of the enzyme protein was presumed as 80 kDa.

Short History on the Research of Inulin Fructotransferase (DFA III-Producing)

Afterwards, there were several reports on the inulin fructotransferase (DFA III-producing) from *Arthrobacter species*. We purified the enzyme produced by *Arthrobacter globiformis* C11-1 [2]. The molecular mass of this enzyme was assumed to be 45 k Da by SDS-

PAGE and 50 k Da by gel filtration (HPLC). From these results, the enzyme was presumed to be a monomer. The enzyme showed maximum activity at pH 5.5 and 55, and the enzyme activity was stable up to 75. Kawamura et al. investigated on the enzyme from *Arthrobacter ilicis* [3]. The molecular mass of this enzyme was presumed to be 27 kDa by SDS-PAGE and 50 k Da by gel filtration. Accordingly, the enzyme was assumed to be a dimer. The enzyme showed maximum activity at pH 5.5 and 60. The enzyme was stable up to 70. Yokota et al. purified the enzyme from *Arthrobacter sp.* H65-7 [4]. The molecular mass of the enzyme was assumed to be 49 kDa by SDS-PAGE and 100 kDa by gel filtration. From these data, the enzyme was presumed to be a dimer. This enzyme showed maximum activity at pH 5.5 and 60. The enzyme was stable up to 70. We reported the enzyme produced by *Arthrobacter sp.* L68-1 [5]. The molecular mass of the enzyme was presumed 43 k Da by SDS-PAGE and 73 k Da by gel filtration. Therefore, the enzyme was assumed to be a dimer. The maximum activity of the enzyme was observed at pH 5.5-6.0 and 55. The enzyme was stable up to 80 for 60 min. This heat-stability was highest among the enzymes reported up to now.

The oligosaccharide DFA III forming enzyme is also produced by other type of bacteria (not belongs to *Arthrobacter*). Kang et al. studied the enzyme produced by *Bacillus sp.* [6]. The molecular mass of the enzyme was assumed to be 62 k Da by SDS-PAGE. This enzyme showed maximum activity at pH 6.0 and 40. The activity of the enzyme was stable up to 60. We studied on the enzyme from *Leifsonia sp.* [7]. The molecular mass of this enzyme was assumed to be 44 k Da by SDS-PAGE, and 74 kDa by gel filtration. Accordingly, this enzyme was presumed to be a dimeric enzyme. The enzyme showed maximum activity at pH 5.0 and 65. The enzyme was stable up to 80. We investigated on the enzyme from *Microbacterium sp.* [8].

The molecular mass of the enzyme was presumed to be 45 kDa by SDS-PAGE, and 46 kDa by gel filtration. Therefore, the enzyme was assumed to be a monomeric enzyme. The enzyme showed maximum activity at pH 6.0 and 60. The enzyme was stable up to 65. Table 1 shows the summary of the properties of oligosaccharide DFA III forming enzyme produced by various microorganisms.

Microorganism	Optimum pH	°C	Heat stability (°C)	Molecular Mass (Kda)		References
				SDS-PAGE	Gel-Filtration	
Arthrobacter ureafaciens	6	50	50		80	[1]
Arthrobacter globiformis C11-1	5	55	75	45	50	[2]
Arthrobacter ilicis OKU17B	5.5	60	70	27	50	[3]
Arthrobacter sp. H65-7	5.5	60	70	49	100	[4]
Arthrobacter sp. L68-1	5.5-6.0	55	80	43	73	[5]
Bacillus sp. snu-7	6	40	60	62		[6]
Leifsonia sp. T88-4	5	65	60	44	74	[7]
Microbacterium sp. S48-1	6	60	65	45	46	[8]

Table 1: Comparison of the inulin fructotransferase (DFA III-producing) from various microorganism.

Cloning of Gene of Inulin Fructotransferase (DFA III-Producing)

Sakurai et al. cloned the enzyme gene of *Arthrobacter sp.* H65-7 [9]. The gene had an open reading frame consisted with 1314 bp. The gene contained a structure of signal peptide (32 amino acids). It was assumed that the mature enzyme was composed of 405 amino acids. The molecular mass of the enzyme was calculated to be 43.4 kDa. The presumed amino acid sequence had a homology (49.8 %) with that of inulin fructotransferase (DFA I-producing) [EC 4.2.2.17] from *Arthrobacter globiformis* S14-3 [10]. The *E. coli* cells carrying the inulin fructotransferase (DFA III-producing) gene of *Arthrobacter sp.* H65-7 produced the active enzyme. Most of the expressed active enzyme existed within the *E. coli* cells.

We cloned the gene of the inulin fructotransferase (DFA III-producing) produced by *Arthrobacter globiformis* C11-1 [11]. The gene encoded an open reading frame consisting 1353 bp. In this gene, the initiation codon was presumed to be an unusual GTG (usually ATG). The gene had a structure of signal peptide (40 amino acids). The molecular mass of the enzyme was estimated as 43.4 kDa from the sequence data. The assumed amino acid sequence of the enzyme had a homology (74.0 %) with that of the *Arthrobacter sp.* H65-7. The deduced amino acid sequence had a homology (45.1 %) with that of inulin fructotransferase (DFA I-producing) from *Arthrobacter globiformis* S14-3 [10]. Figure 2 shows the homology of these presumed amino acid sequences. The cloned gene from *A. globiformis* C11-1 was expressed in the host vector system of E. coli (pUC 119, *E. coli* JM109). In this case, the active enzyme was expressed in both in a cell free extract and the culture supernatant. The ratio of the total activity expressed (cell free extract: culture supernatant) was 61: 39.

Production of DFA III Using an Immobilized Enzyme or a Membrane Reactor

Jahnz et al. reported on an immobilization of inulin fructotransferase (DFA III-producing) [12]. They used calcium alginate as a carrier of immobilization. The prepared immobilized enzyme had an activity of 196 U per gram gel.

Figure 2: Alignment of the deduced amino acid sequences of inulinfructotransferases. ; C11-1, deduced amino acid sequence of inulin fructotransferase (DFA III-producing) from *Arthrobater globiformis* C11-1; H65-7, deduced sequence of inulin fructotransferase (DFA III-producing) from *Arthrobacter sp.* H65-7; S14-3, deduced sequence of inulin fructotransferase (DFA I-producing) from *Arthrobacter globiformis* S14-3. The identical residues are presented by white letters in black boxes.

Bo et al. reported on an enzymatic membrane reactor (EMR) for the production of oligosaccharide DFA III [13]. In this system, ultrafiltration membrane was employed and the reuse of the DFA III forming enzyme was performed. The purity of produced DFA III was improved to 95 %. Using this EMR system, the large scale production of the DFA III was able to achieve.

Functionality of Oligosaccharide DFA III

Suzuki et al. reported on the special functionality of the oligosaccharide DFA III using rats [14]. They performed an experiment using a rat of 5 week age and weight about 100 g. The four groups of the rats were prepared. The groups were given a diet containing DFA III or raffinose or fructo-oligosaccharide or no oligosaccharide (control). The group of the rats given DFA III was most effective on the absorption of calcium from diet. Therefore, the combination of oligosaccharide DFA III and calcium promotes the absorption of calcium from intestines. The calcium is absorbed from a tight-junction of the intestines. The oligosaccharide DFA III has an effect on the tight-junction and promotes the assimilation of calcium. Further-more, the organic acids are formed by intestinal microorganism from DFA III. These organic acids dissolve calcium and promote the absorption of calcium from intestines.

Industrialization of Oligosaccharide DFA III

A beet sugar company in Hokkaido, Japan started a development for the industrial production of DFA III on a commercial basis around 1994. The company employed the inulin fructotransferase (DFA III-producing) produced by *Arthrobacter sp.* H65-7 [4]. As an original material, the inulin extracted from chicory was used. The inulin was imported from Germany. The sugar company produced the pure oligosaccharide DFA III powder in large scale. Using this powder, a health food maker in Japan developed a supplement containing DFA III and minerals (Fe, Ca, Mg, and so on). The sales of the supplement were started in 2004, in Japan. Now, the supplement using oligosaccharide DFA III is sold in a pharmacy and a convenience store, in Japan.

Conclusion

The oligosaccharide DFA III producing enzyme was discovered in 1973 by Uchiyama et al. [1]. This is the beginning of the basic research

of the enzyme. The industrial production of DFA III was started in 2004 in Japan. Therefore, it took more than 30 years from the start of the basic research to the industrial production. During this period, there were many researchers and technical specialists concerned the development for the industrial production of the oligosaccharide DFA III. Please notice that it takes a very long time for the birth of a new practical technique.

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