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Short communication

Difructosan anhydrides III preparation from sucrose by coupled enzyme reaction

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ABSTRACT

Difructosan anhydrides III (DFA III) preparation was usually obtained by inulin hydrolysis with inulin fructotransferase (IFTase). The fructofuranosidic linkages of inulin were the same as fructooligosaccharides (FOS), which was synthesized by sucrose with fructosyltransferase (FTase). FOS was mainly composed of 1-kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4), and nystose was observed to be the smallest substrate for IFTase to synthesize DFA III. So sucrose, much cheaper than inulin, was considered to produce DFA III by coupled FTase and IFTase reaction. DFA III yield was obtained about 100 mg/g (DFA III weight/sucrose weight) through this method. The results demonstrated the high potential of the coupled enzyme reaction as a novel DFA III producing method.

1. Introduction

DFA III is a cyclic disaccharide consisting of two fructose units linked at their reducing carbons. DFA III was produced from inulin by the exo-acting intramolecular transfructosylation of inulin fructotransferase (DFA III-producing) (IFTase, EC 4.2.2.18). IFTase was firstly described in *Arthrobacter ureafaciens* (Chiyama, Niwa, & Tanaka, 1973). Subsequently, there have been several reports on the IFTase from *Arthrobacter* species (Haraguchi, Yoshida, & Ohtsubo, 2005; Yokota et al., 1991) and has meanwhile been found in various other bacteria (Cho, Lee, Hwang, Jang, & Lee, 1997; Haraguchi, Yoshida, & Ohtsubo, 2006). DFA III is non-reducing sugar which has half the sweetness and 1/15 calories of sucrose (Kikuchi et al., 2004). DFA III was found to increase the absorption of calcium in small and large intestines of rats (Suzuki, Hara, Kasai, & Tomita, 1998), and low dose of DFA III had a stimulating effect on calcium absorption in humans (Shigematsu, Okuhara, Shiomi, Tomita, & Hara, 2004). More interests in the enzymatic production of DFA III have been stimulated by its potential functions as food additive.

The present studies mainly hydrolyze inulin to produce DFA III with IFTase. DFA III was produced by a heat stable inulin fructotransferase (DFA III-producing) from *Arthrobacter pascens* T13-2 and *Arthrobacter sp.* L68-1 (Haraguchi, Yamanaka, & Ohtsubo, 2002; Haraguchi et al., 2005). DFA III was obtained by genes encoding an inulin fructotransferase (DFA III-producing) from *Arthrobacter sp.* H65-7 and *Arthrobacter globiformis* C11-1 (Haraguchi, Mori, & Hayashi, 2000; Sakurai, Yokota, & Tomita, 1997). It is valuable to investigate other cheap substrates to substitute inulin for DFA III production, because inulin is much expensive. FOS can be synthesized by sucrose with fructosyltransferase (FTase, EC 2.4.1.19), in which 1–3 fructose units are bound at the β-2,1 position of sucrose, and it is mainly composed of 1-kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4). FOS could be prepared by levansucrase from *Microbacterium laevaniformans* ATCC15953 (Park et al., 2003) and β-fructofuranosidase from *Aspergillus niger* IMI303386 (Nguyen, Rezessy-Szabó, Bhat, & Hoschke, 2005). As the fructofuranosidic linkages of FOS were the same as inulin and nystose was observed t o be the smallest substrate for IFTase (Zhao et al., 2010), the report was explored to utilize sucrose as a substrate to produce DFA III through coupled enzyme (FTase and IFTase) reaction.

2. Experimental

2.1. Materials and reagents

DFA III, 1-kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4) were purchased from Wako Pure Chemical Industries (Osaka, Japan). FTase and IFTase were obtained from the extract of *A. niger* AS0023 and *Arthrobacter aurescens* SK 8.001, the bacteria were maintained by our laboratory (L'Hocine, Wang, Jiang, & Xu, 2000; Meng et al., 2010). All other chemicals were of analytical grade and used as received.

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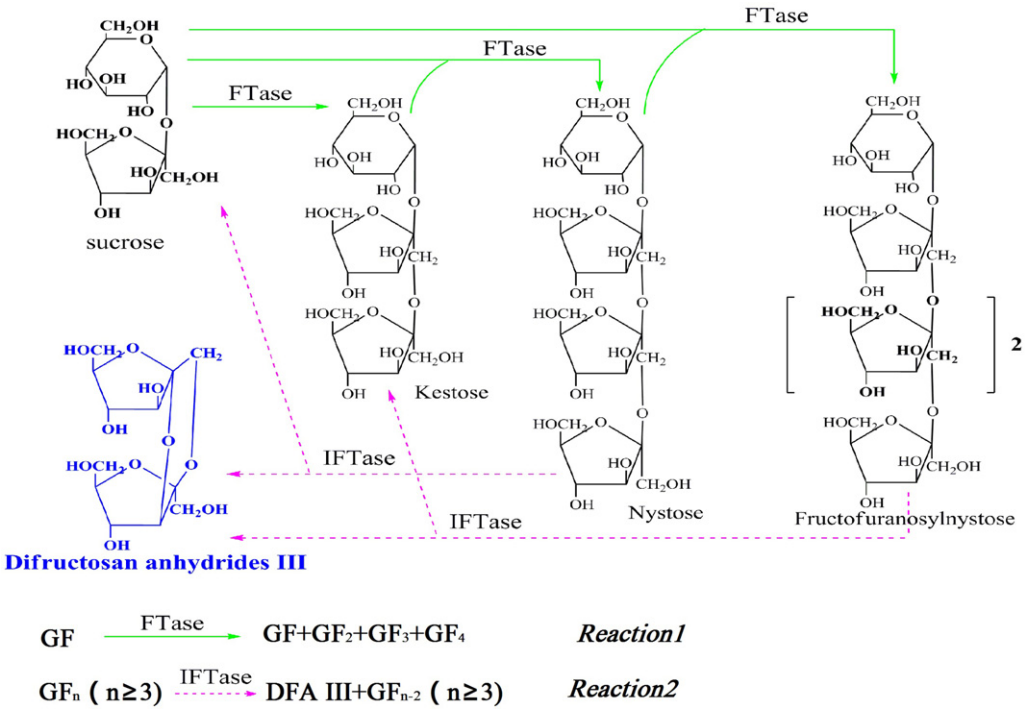

Fig. 1. Mechanism of coupled enzyme reaction. G—glucose, GF—sucrose, GF_2—kestose, GF_3—nystose, GF_4—fructofuranosylnystose, *n*—the number of fructose.

2.2. The mechanism of coupled enzyme reaction

The mechanism of coupled enzyme reaction was shown in (Fig. 1). It revealed the reaction mechanisms of converting sucrose to FOS by FTase and transforming GF_n ($n \geq 3$) into DFA III by IFTase, which was respectively called Reaction 1 and Reaction 2. In Fig. 1, it was clearly seen that GF could be converted to GF_2, GF_3 and GF_4 by FTase due to Reaction1; meanwhile, GF_3 and GF_4 were hydrolyzed to DFA III, GF and GF_2 by IFTase due to Reaction 2. In addition, in the cyclic coupled enzyme reaction system, consumption of GF_3 and GF_4 with IFTase in Reaction 2 could alleviate the accumulation of products of FTase in Reaction 1. Meanwhile, GF and GF_2 regenerated in Reaction 2 could be utilized as substrates for FTase in Reaction 1. This method could make the experimental operations recycle.

2.3. DFA III production by coupled enzyme reaction

The reaction solution was kept at a constant volume of 100 ml in all the experiments, which contained enzyme qualities (10 mg FTase and 10 mg IFTase) under various reaction conditions. Reaction conditions are pH 4.0–8.0, temperature for 35–70 °C, substrate concentrations for 100–600 g/l (m/v).

2.4. HPLC conditions

For determination of DFA III, an Agilent HPLC system (Agilent Technologies, Inc., Santa Clara, CA) equipped with an Agilent 1200 quaternary pump, thermoset column compartment and 1200 series diode array detector was used. Chemstation software (Agilent) was used for data acquisition and processing on a personal computer. Aliquots of 10 μl were injected onto a Thermo scientific amino column (4.6 mm × 250 mm) and Refractive Index Detector was used. Running conditions of the analysis were as follows: column temperature, 35 °C; mobile phase, acetonitrile:water (65:35); flow rate, 1 ml/min.

3. Results and discussion

3.1. Production of DFA III by coupled enzyme reaction with sucrose

The HPLC analysis could be used for rapid determination of DFA III due to the second peak or the relative retention time of 4.112 min (Fig. 2). The first peak was solvent, and the surplus peaks were G, GF, GF_2, GF_3 and GF_4 from 4.563 min to 7.584 min. The DFA III yield could reach about 100 mg/g (DFA III weight/sucrose weight) after reaction for 36 h, which was the first report of producing DFA III with sucrose as a substrate through the coupled enzyme reaction. Compared with DFA III preparation with IFTase from *Arthrobacter sp*. H65-7 (Yokota et al., 1991), in which 300 mg/ml of the inulin

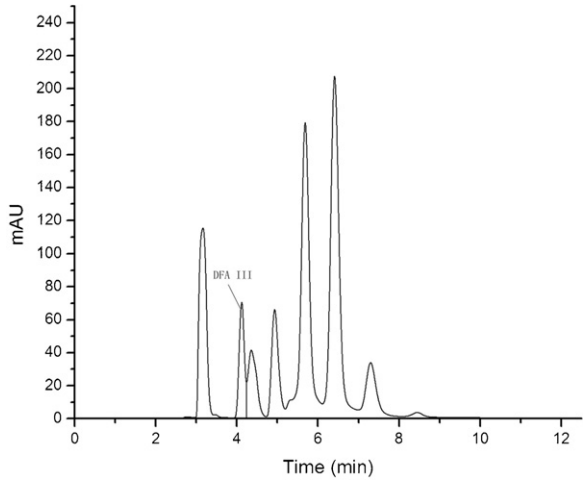

Fig. 2. HPLC profile of DFA III production by the coupled enzyme reaction. The HPLC conditions were as follows: mobile phase, acetonitrile:water (65:35); flow rate, 1 ml/min; column, Thermo scientific amino column (4.6 mm × 250 mm); column temperature, 35 °C; and Refractive Index Detector. The peak at 4.112 min shows the eluting peak of DFA III.

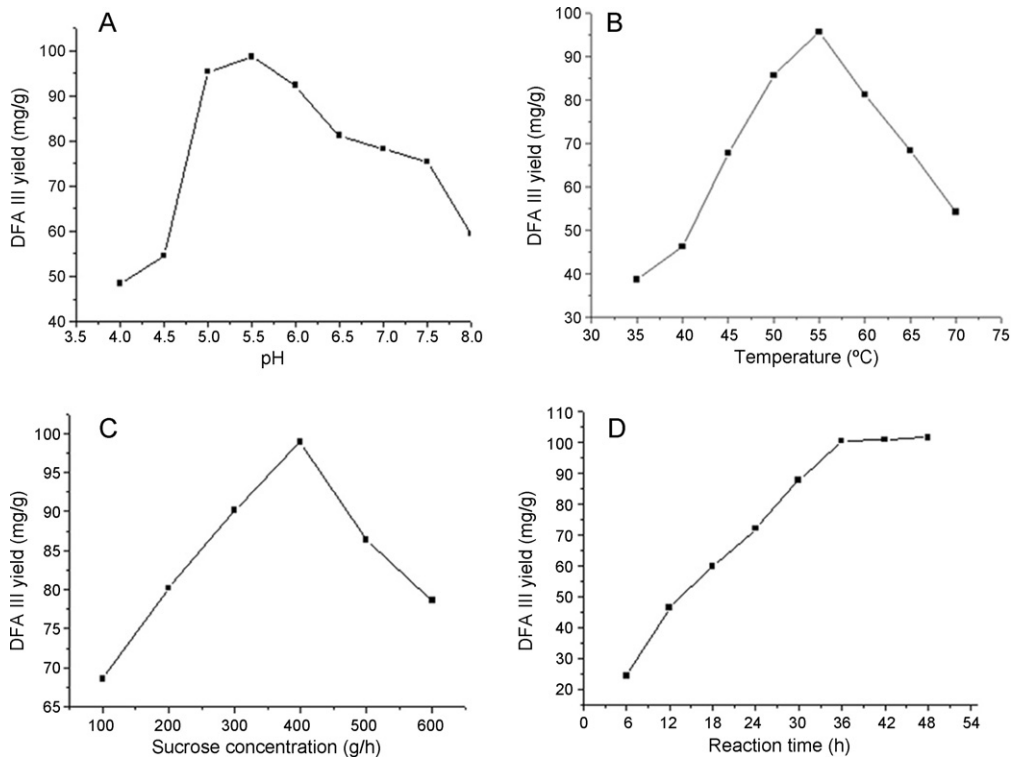

Fig. 3. Optimum reaction conditions of the coupled enzyme reaction. The DFA III yield (mg/g) was defined as DFA III weight/sucrose weight. A–D shows optimum pH 5.5, temperature 55 °C, substrate concentration 400 g/l and reaction time 36 h, respectively.

could be converted into 237 mg/ml of DFA III after incubation for 4 h, which means DFA III yield could attain about 80% in such circumstances, our results showed DFA III yield was much lower. This may not be surprising because only the wild-type proteins were considered here and the substrate was FOS but not inulin of the suitable substrate for IFTase.

3.2. Determination of the coupled enzyme reaction conditions

FTase was obtained from *A. niger* AS0023, and its optimum pH and temperature was found to be pH 5.8 and 50 °C (L′Hocine et al., 2000). The soil bacterium *A. aurescens* SK 8.001 produced IFTase, which showed maximum activity at pH 5.5 and temperature 60–70 °C (Meng et al., 2010). We determined conditions of the coupled enzyme reaction for varying substrate concentrations, pH, temperature and reaction time. The results were shown in (Fig. 3). The maximum DFA III yield was obtained at pH 5.5 (Fig. 3A), and this pH value was the same with IFTase and lower than FTase. The coupled enzyme reaction exhibited optimum temperature at 55 °C (Fig. 3B), which was a little higher than FTase and lower than IFTase. As shown in Fig. 3C, the maximum DFA III yield was obtained when the concentration was 400 g/l, which was lower than that of FTase (500 g/l). Fig. 3D shows that reaction time was 36 h, the DFA III yield was obtained the maximum (100 mg/g) at the conditions of pH 5.5, temperature 55 °C and sucrose concentration 400 g/l. So the optimum reaction conditions of the coupled enzyme reaction were determined (data not shown). These conditions were then applied in the process of reaction. The results showed that the application of the coupled enzyme reaction to produce DFA III would be advantageous.

4. Conclusions

In summary, we discovered that sucrose could be catalyzed by the coupled enzyme reaction to produce DFA III. In this experiment, 100 mg/g of DFA III was obtained and the optimum conditions of the coupled enzyme reaction were determined. Although the details of the mechanism of catalysis remained to be uncovered, the present observations could extend the actual application of the coupled enzyme reaction. This is of special significance because the coupled enzyme reaction has been scarcely reported in producing DFA III. DFA III yield was low. This might not be surprising because only the wild-type proteins were considered here and not all FOS components were the suitable substrate for IFTase. Since sucrose is much lower than inulin, the future works will improve the ability of IFTase to hydrolyze FOS by genetic engineer modification of the strain. Using this method, the coupled enzyme reaction could convert sucrose into DFA III with target yield 40–50% and develop a process to make DFA III from sucrose directly instead of inulin.

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