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Optimal expression and characterization of a fusion enzyme having dextransucrase and dextranase activities

Hwa-Ja Ryu[a,1], Xingji Jin[b,1], Jin-Ha Lee[c], Hye-Jin Woo[a], Young-Min Kim[d], Ghahyun J. Kim[e], Eun-Seong Seo[a], Hee-Kyoung Kang[a], Jongho Kim[f], Dong-Lyun Cho[g], Atsuo Kimura[h], Doman Kim[a,*]

[a] School of Biological Sciences and Technology and The Research Institute for Catalysis, Chonnam National University, Gwang-Ju 500-757, Republic of Korea
[b] GenoFocus, Inc., A Daejeon BioVenture Town, 461-8 Jeonmin-Dong, Yusung-gu, Daejon 305-811, Republic of Korea
[c] Dental Science Research Institute, Brain Korea 21 Project, School of Dentistry Chonnam National University, Gwang-Ju 500-757, Republic of Korea
[d] Jeonbuk Branch Institute, Korea Research Institute of Bioscience and Biotechnology, Jeonbuk 580-185, Republic of Korea
[e] Major in Biochemistry and Cell Biology, University of California-San Diego, La Jolla, CA 92093, USA
[f] School of Applied Chemical Engineering and The Research Institute for Catalysis, Chonnam National University, Gwang-Ju 500-757, Republic of Korea
[g] Center for Functional Nano Chemicals, Chonnam National University, Gwang-Ju 500-757, Republic of Korea
[h] Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

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ABSTRACT

Long isomalto-oligosaccharides (IMOs) were generated via an engineered fusion enzyme of dextransucrase and dextranase (DSXR). To increase the expression level, response surface methodology (RSM) was utilized for optimization of protein expression conditions for enhancement of protein production by the effects of three-level-three-factors and their mutual interaction in *Escherichia coli*. Seventeen experiments were designed and conducted for investigation of cell density to start induction, induction temperature, and induction time. Optimal induction conditions included a cell density to start induction (A_{600}) of 0.76 at 12.16 °C for 18 h for dextransucrase activity and a cell density to start induction (A_{600}) of 0.75 at 10.5 °C for 20.9 h for dextranase activity. The produced dextransucrase or dextranase activity was obtained at 120.1 ± 7.2 or 871 ± 58 U, respectively, from 1 L cultures.

1. Introduction

Prebiotics are non-digestible carbohydrates that beneficially influence the host through improvement of intestinal microbial balance in the human colon [1]. Among a variety of oligosaccharides showing evidence of prebiotic function [2], isomalto-oligosaccharide IMO is a well-known saccharide, particularly in Japan, where demand is approximately 15,000 tons per year [3]. IMO is defined as a saccharide that contains 40% α-(1–6) glucosidic linkages among its total linkages [4]. Commercially available IMOs with degrees of polymerization (DP) ranging from 2 to 6 are generated from corn starch via a two-step enzymatic hydrolysis and transglucosylation process from starch [3,4]. Larger IMOs are absorbed at a significantly lower amount, and remain in the colon longer due to persistence against glucosidase-mediated hydrolysis [5]. Thus, IMOs with larger DP display a greater prebiotic effect toward *Bifidobacteria* than small IMOs [6]. IMOs that contain nearly exclusive α-(1–6) glucosidic bonds can be produced using two enzymatic processes: the first is a dextransucrase catalyzed transfer of glucosyl residues from sucrose to acceptors, and the second is a dextranase-mediated hydrolysis of dextran. It is suggested that dextran hydrolyzates of dextranase would be used as acceptors for production of IMOs by acceptor reactions: transfer of a glucosyl unit from sucrose to another carbohydrate. We previously reported on a fusion enzyme, designated DXSR (a fusion enzyme of dextranase–dextransucrase), which incorporates α-(1–6) glucan hydrolyzing dextranase from *Arthrobacter oxydans* and α-(1–6) glucan synthesizing dextransucrase from *Leuconostoc mesenteroides* DSRBCB4 [7]. Unlike the mixture of each dextranase and dextransucrase, DXSR increases the reaction velocity for IMO production and generates mostly α-(1–6) glucosidic linkage IMOs with high DP2–DP10 [7]. This enzyme is more cost-effective than individual production of both dextranase and dextransucrase. For the improvement of productivity of dextransucrase, dextranase and IMOs, the new fusion protein, DSXR, was constructed. Unlike the order of dextranase–dextransucrase in DXSR, the new fusion gene, which has been designated as *dsxr*, was sequentially composed of the dextransucrase-encoding gene (*dsrBCB4*, GenBank accession no. DQ497800) and the dextranase-encoding gene (*dex2*, GenBank accession no. DQ497801). The process for enzyme expression was optimized using a combination of factorial design and response sur-

* Corresponding author. Tel.: +82 62 530 1844; fax: +82 62 530 1949.
E-mail addresses: dmkim@jnu.ac.kr, dmkim@chonnam.ac.kr (D. Kim).
[1] These authors contributed equally to this work.

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face methodology (RSM). RSM is an effective statistical tool that is widely applied in the optimization of fermentation processes, which includes experimental design, optimization of biomass production, and culture conditions. It has several advantages that includes less experiment numbers, suitability for multiple factor experiments, searching for relativity between factors, and finding of the most suitable condition, forecasting response and minimizing the error in determining the effect of parameters. RSM contains many reported designs such as Box–Behnken design, Graeco–Latin design and central composite design (CCD) [8].

This study was undertaken in order to conduct a component optimization for each activity of DSXR under expression conditions. Components that were investigated included starting cell density, incubation temperature, and culture time using central composite design with the simultaneous effect of three independent variables for facilitation of industrial-scale production. Biochemical properties of expressed DSXR were compared with parent enzymes and DXSR.

2. Materials and methods

2.1. Construction of expression vectors for DSXR

All polymerase chain reaction (PCR) procedures were conducted using Ex*Taq* DNA polymerase (Takara, Kyoto, Japan). The *dsxr* gene was composed of the *dsrBCB4* gene (4425 bp) from *L. mesenteroides* B-1299CB4 and the *dex2* gene (1861 bp) from *A. oxydans* [7]. For insertion of the *dsrBCB4* gene into the pRSETC vector (Invitrogen, Carlsbad, CA, USA), two primers (sense primer, 5′-ATTTTATCTCGAGTTATGCTGTCTATGA-3′, antisense primer 5′-GGCTTTTTTTAGTTAAGATCTTGAGACA-3′) were designed based on the DNA sequence of *dsrBCB4* (accession No. AF030129). *Xho*I and *Bgl*II sites were introduced to the 5′- and 3′-termini of the *dsrBCB4* gene (underlined), respectively. The amplified 4.47-kb DNA fragment was inserted into pRSETC via the *Xho*I and *Bgl*II sites for construction of pRSETC-*dsrBCB4*. Two primers (sense primer, 5′-GATCGATGGATCAGATCTAAGCATTAC-3′, antisense primer 5′-ATCAAGCTT CGAATTCCATGGTACCC-3′) of the 5′- and 3′-termini of the *dex2* gene were designed for insertion of *dex2* into the plasmid; these primers harbored a *Bgl*II and *Nco*I restriction site (underlined), respectively, based on the *dex2* gene (GenBank accession no. DQ497801). The amplified 1.88-kb DNA fragment of *dex2* was inserted into pRSETC-*dsrBCB4* via the *Bgl*II and *Nco*I sites for construction of pRSETC-*dsxr*. Other genetic manipulations were conducted in accordance with the method described by Sambrook and Russell [9]. Sequencing of amplified DNA and pRSETC-*dsxr* were repeated twice by the Korea Basic Science Institute (KBSI, Gwangju, Korea), and no differences in the sequence were detected.

2.2. Expression of DSXR and investigation of variable induction conditions

DSXR was generated in *Escherichia coli* BL21(DE3)pLysS (Invitrogen, Carlsbad, CA, USA) transformants harboring pRSETC-*dsxr*. The *E. coli* transformant was grown in 100 mL LB medium containing 50 μg mL^{-1} of ampicillin in a 250-mL flask at 37 °C until $A_{600\,nm}$ reached approximately 0.5, 0.7 or 0.9, and the proteins were induced with 1 mM of lactose [10]. The effect of induction time within a range of 12–24 h and the effect of induction temperature (1, 10.5, or 20 °C) was experimentally investigated.

Cell-free extract was prepared by sonication using a Sonifier 250 (Branson Ultrasonics, Danbury, CT, USA) at 4 °C. Protein concentration was determined via the Bradford method [11] using bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO, USA) as the standard.

2.3. Optimization procedure and experimental design

A three-level Box–Behnken design with three-factors was applied for the DSXR optimization procedure using Design Expert 6 software (Stat-Ease, Minneapolis, MN, USA), including five replicates at the central point, which were utilized in the fitting of a second-order response surface. Several factors, including starting cell density (X_1), induction time (X_2), and induction temperature (X_3) were utilized in preparation of each of the 17 culture conditions summarized in Table 2. Optimization was conducted using a desirability function for determination of levels of X_1, X_2, and X_3 on dextransucrase and dextranase activities (Y). A total of 17 experiments were required for determination of the 10 coefficients of the model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

in which Y is the predicted response; 0 is intercept, 1, 2, and 3 are linear coefficients; 11, 22, and 33 are squared coefficients; 12, 13, and 23 are interaction coefficients. Once an appropriate model was obtained, this model was used to determine the predicted optimum conditions for the process.

2.4. Biochemical properties of DSXR

For analysis of enzyme activity, sucrose or dextran T2000 (Sigma–Aldrich) was utilized as the substrate for dextransucrase or dextranase, respectively. A reaction mixture consisting of an adequate concentration of enzyme, 200 mM sucrose, or 1% (w/v) dextran T2000 in 20 mM sodium acetate buffer (pH 5.8) was incubated for 5–10 min at 28 °C. The enzymatic reaction was halted by heating for 5 min, and the amount of liberated fructose or reducing sugar was determined by thin layer chromatography (TLC) [12] or the copper–bicinchoninate method [13]. One unit of dextransucrase or dextranase was defined as the quantity of enzyme required to release 1 μmol of fructose from sucrose or isomaltose equivalent from dextran per min under the assay conditions [7].

Optimal temperature and thermal stability of DSXR (dextranase equivalent activity of 0.24 U mL^{-1}) on IMO production was assessed as follows. DSXR was incubated for 10 min at various temperatures (ranging from 4 to 60 °C) in 20 mM sodium acetate buffer (pH 5.8). Thermo-stability of DSXR activity for IMO production was measured by incubation with DSXR 3 h at various temperatures, and was followed by the reaction with 200 mM sucrose. To understand the effect of pH, DSXR (dextranase equivalent activity of 0.24 U mL^{-1}) was incubated for 10 min with 200 mM sucrose, which was adjusted to various pH values (pH 3.5–9.5). With regard to the pH stability of DSXR for IMO production, DSXR was incubated for 3 h in 20 mM sodium acetate buffer at various pH values, followed by reaction with 200 mM sucrose at 28 °C. Each residual activity of DSXR for IMO production was determined by measurement of the total IMO via TLC [14].

2.5. Synthesis of oligosaccharides

To investigate IMO production by DSXR, DSXR (8.7 U for dextranase activity and 1.2 U for dextransucrase activity) and 100 mM sucrose were incubated at 28 °C in sodium acetate buffer (pH 5.8) for 24 h. Reaction aliquots were subjected to high performance liquid chromatography (Shimadzu, Kyoto, Japan) under the following conditions: TSK-Gel Amide 80 (4.6 mm × 250 mm I.D.; Tosoh, Tokyo, Japan); mobile phase, CH_3CN:H_2O (53:47, v/v); flow rate, 0.5 mL min^{-1}; temperature, 24 °C; detection, RI detector (RID-10A model, Shimadzu). A series of IMOs were used as standard saccharides.

3. Results and discussion

3.1. Characterization of DSXR and RSM optimization of culture conditions for DSXR

Culture conditions for promotion of optimal DSXR production were established. From preliminary experiments for evaluation of the type of inducer, concentration of inducer, inoculum density to start induction, induction time, and induction temperature, three factors (inoculums density to start induction, induction time, and induction temperature) were selected for optimization of DSXR production (data not shown). Specifically, optimal conditions for production of DSXR were as follows: inoculum density to start induction ($A_{600\,nm}$), 0.5–0.9, induction time, 12–24 h, and induction temperature of 1–20 °C. Optimization of enzyme activity was achieved by employment of a Box–Behnken design for the experiments [15]. Selected culture conditions, including inoculums density to start induction (X_1), induction time (X_2), and induction temperature (X_3), were investigated. RSM was used to study the interaction of these variables in relation to DSXR production. A total of 17 experiments were performed with different combinations of these factors (Table 1). The design matrix and corresponding results of RSM experiments, along with the mean predicted values, are shown in Table 2. DSXR production varied with changes in starting cell density, cultural induction time, and cultural induction temperature. As ascertained from the central points of the corresponding contour plots (Fig. 1), the conditions of the three variables that proved optimal for dextransucrase activity included starting cell density (OD = 0.76), induction time (18 h), and cultural induction temperature (12.16 °C). Dextransucrase equivalent activity was measured at 115.8 U L^{-1}. In the case of dextranase, the three variables included starting cell density (OD = 0.75), cultural induction time (20.9 h), and cultural induction temperature (10.5 °C).