Journal of Molecular Catalysis B: Enzymatic 128 (2016) 27–38

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Synergistic effect between the recombinant exo-inulinase and endo-inulinase on inulin hydrolysis

Yuan Liu [a], Shun-Hua Zhou [a], Yu-Rong Cheng [b], Zhe Chi [a], Zhen-Ming Chi [a], Guang-Lei Liu [a,*]

[a] Ocean University of China, Yushan Road, No. 5, Qingdao, Shandong 266003, People's Republic of China
[b] Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Songling Road, No. 189, Qingdao, Shandong 266101, People's Republic of China

ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 27 February 2016
Accepted 16 March 2016
Available online 17 March 2016

Keywords:
Synergistic effect
Exo-inulinase
Endo-inulinase
Inulin
Inulo-oligosaccharides

ABSTRACT

An exo-inulinase gene from *Kluyveromyces marxianus* and an endo-inulinase gene from *Aspergillus niger* were cloned and expressed in *Yarrowia lipolytica,* respectively. Then, the purified recombinant exo-inulinase (rEXINU) and endo-inulinase (rENINU), either individual or combination with another, were used for hydrolysis of inulin and inulin in tuber meal of Jerusalem artichoke. After the analysis of the dynamic hydrolysis process, it was found that the rEXINU and the rENINU acted synergistically with each other, and the maximum degree of synergistic effect on the level of fructose formation (DSE_{Fru}) could be obtained with a molar ratio of the rEXINU to the rENINU at 1:1. Furthermore, the investigation of the hydrolysis product in the synergistic hydrolysis process showed that a wide range of oligosaccharides were first generated and then converted to fructose. Based on the enzyme kinetics, substrate specificity and product inhibition of the rEXINU and the rENINU, a synergism mechanism of the them for the inulin hydrolysis was proposed. During the process of the synergistic action, the rENINU preferentially degraded the inulin with high DP, but was inhibited by the produced oligosaccharides. Then the released oligosaccharides which were the optimum substrates of the rEXIUN were rapidly hydrolyzed by the enzyme to produce fructose and glucose. Meanwhile, this process relieved the inhibition of the produced oligosaccharides to the rENINU. Besides, in the synergistic hydrolysis of the inulin in tuber meal of Jerusalem artichoke, the depolymerization of the rENINU could result in the increase of the subtrate solubility to improve the hydrolysis efficiency. Therefore, this kind of the synergistic effect between the exo-inulinase and the endo-inulinase may provide a potential driving force in the industrial application of inulin and inulin-containing materials.

1. Introduction

Inulin, a reserve carbohydrate in plants such as Jerusalem artichoke, chicory, dahlia, and yacon, consists of linear chains of β-2,1-linked D-fructofuranose residues terminated by a glucose residue [1]. The degree of polymerization (DP) of these fructose chains varies and ranges from 2 to 60, and an inulin containing maximally ten monomers is also referred to as an inulo-oligosaccharide [2]. The main hydrolysis product of inulin is fructose, which is not only a safe alternative sweetener in the food or beverage industry, but also considered to be a desirable carbon source in the fermentation industry [3]. Recently, as a result of the success in the breeding and extension of the new Jerusalem artichoke cultivars with good qualities, such as no farmland occupation, multiple resistances, stress tolerances, and high yield, the inulin-containing materials have already been recognized as a competitive and alternative biomass resource compared with the starchy materials from food grains, and have a potential to be extensively used for biotransformation into fructose syrup, ethanol, lipids, pullulan, citric acid and other chemicals [4–9]. However, the improvement of the inulin hydrolysis efficiency is still one of the most important and urgent issues in the inulin-containing materials processing industry.

The enzymatic degradation of biomacromolecules often involves a combination of exo-acting and endo-acting depolymerizing enzymes. Inulin is degraded mainly by inulinases, which was divided into exo-inulinases (EC 3.2.1.80) and endo-inulinases (EC 3.2.1.7) according to their hydrolysis pattern. The exo-inulinases catalyze the removal of the terminal fructose residue from the non-

* Corresponding author.
E-mail address: liugl@ouc.edu.cn (G.-L. Liu).

http://dx.doi.org/10.1016/j.molcatb.2016.03.005
1381-1177/

Table 1
The primers used in this study.

Primer	Sequence	Use
Exosfi	5′-GGCCGTTCTGGCCTCAGTTATCAATTACAAGAGAGATGGTGACAGC-3′ (the underlined bases encode *Sfi*I site)	Amplification of the *Exoinu* for the plasmid constriction
Exobam	5′-GGATCCTCA**ATGGTGATGGTGGTGATG**AACGTTAAATTGGGTAACG-3′ (the bold bases encode *BamH*I site and the bold bases encode 6 × His)	
Endosfi	5′-GGCCGTTCTGGCCCAGTCTAATGATTACCGTCC-3′ (the underlined bases encode *Sfi*I site)	Amplification of the *Endoinu* for the plasmid constriction
Endokpn	5′-GGTACCTTA**ATGATGATGATGATGATG**TTCAAGTGAAACACTCCGC-3′ (the bold bases encode *BamH*I site and the bold bases encode 6 × His)	
1317s	5′-CAATGAAGCTCGCTACCGCC-3′	Amplification for confirmation of integration of the target gene
1317a	5′-ACAGGCCATGGAGGTACCGG-3′	

reducing ends of the inulin chains, whereas the endo-inulinases attack inulin chains at random positions to yield fructooligosaccharides [6]. Invertases (EC 3.2.1.26) also have an exo-inulinase activity, and specially hydrolyze fructooligosaccharides with a low degree of polymerization values [10]. In our previous study [4], after an endo-inulinase gene from *Arthrobacter* sp. was expressed in *Saccharomyces* sp. W0, the inulin utilization and ethanol fermentation of the recombinant yeast D5 obtained were improved prominently due to the collaboration between the heterologous endoinulinase and the inherent invertase. In another study [7], a crude inulinase mixture from *Aspergillus niger* TISTR 3570 and *Candida guilliermondii* TISTR was used for hydrolyzing inulin into fructose, and the enzyme mixture provided a better combination of endo- and exo-inulinase activities than that of the crude extracts of either the mold or the yeast individually. Subsequently, it was also demonstrated that the inulin was hydrolyzed high-efficiently by a mixture of invertase, endo- and exo-inulinases produced by five fungi of *Penicillium* and *Aspergillus* [11]. Although the previous studies showed that the coexpression or mixture of different inulinases was necessary for efficient degradation of inulin, they have failed to investigate the synergistic cooperation of the purified inulinases, and neglected to quantify the synergistic effect, investigate the synergistic kinetics, and explore the synergism mechanism.

On the other hand, the numerous studies on glycan degradation by using the enzymes such as exo-chitinase/endo-chitinase [12], β-D-xylosidase/α-L-arabinofuranosidase [13], α-amylase/amyloglucosidase [14,15], and β-glucosidase/endo-glucanase/exo-glucanase [1,16–18] have been done in order to elucidate their synergistic actions. However, so far little has been known about the synergistic mechanism of exo- and endo-inulinase.

In the present study, a recombinant exo-inulinase and a recombinant endo-inulinase were used for the synergistic hydrolysis of inulin. Finally a synergism mechanism of the exo- and endo-inulinases for inulin hydrolysis was proposed.

2. Materials and methods

2.1. Strains, media and plasmids

Kluyveromyces marxianus KM-0 strain (collection number 2E001023 at the Marine Microorganisms Culture Collection of China, MCCC) which can produce high level of an exo-inulinase [19] was used for the exo-inulinase gene (*Exinu*) cloning. *A. niger* F4 strain (collection number 2E001022 at MCCC) isolated from a mangrove system in Hainan Province of China was used for the endo-inulinase gene (*Endoinu*) cloning. *Escherichia coli* DH5α [F^-, ϕ80*lacZΔM15*, *Δ*(*lacZYA-argF*), U169, *endA1*, *recA1*, *hsdR17* (*rk*$^-$, *mk*$^+$), *supE44*, λ$^-$, *thi-1*, *gyrA96*, *relA1*, *phoA*] (Tiangen Biotech, Beijing, China) was used to amplify the plasmids carrying the endo- and exo- inulinase genes. The uracil mutant *Yarrowia lipolytica* Po1h [*MatA*, *ura3-302*, *xpr2-322*, *axp1-2*; *Ura*$^-$ *ΔAEP*,*ΔAXP*, *Suc*$^+$][20] was used to express the heterologous inulinase genes. The *E. coli* transformants were grown in a Luria-Bertani (LB) medium with 100.0 μg/mL of ampicillin or 30 μg/mL of kanamycin. Yeast strains were grown in a Yeast extract-peptone-dextrose (YPD) medium containing 10 g/L yeast extract, 20 g/L polypeptone and 20 g/L glucose. The yeast transformants were grown in a Yeast extract-Nitrogen-Base (YNB) medium containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 10 g/L glucose, 5 g/L ammonium sulfate, and 25 g/L agar. To produce heterologous protein by yeast transformants, an inulinase production medium containing 20 g/L inulin for the exo-inulinase production or 20 g/L sucrose for the endo-inulinase production, 1.32 g/L yeast extract, 1.32 g/L NH_4Cl, 0.32 g/L KH_2PO_4, 0.24 g/L $MgSO_4{\cdot}7H_2O$, 0.33 mg/L thiamine, and pH 5.5 was used. A plasmid pINA1317 used to express heterologous genes in *Y. lipolytica* Po1h was kindly supplied by CBAI, INAPG, 78850 Thiverval-Grignon, France.

2.2. Inulo-oligosaccharides, inulin and Jerusalem artichoke

Inulo-oligosaccharides (IOS) composed of a range of oligosaccharides from DP2 to DP9 were purchased from Pioneer Biotech Co., Ltd., Xian, China. Inulin with an degree of polymerization of 36 was purchased from Sigma-Aldrich, St. Louis, MO, USA (product code I3754, inulin from dahlia tubers). The tuber meal (contained 70% inulin) of Jerusalem artichoke, which was processed by peeling, slicing, drying, milling, was kindly supplied by Prof. Liu from Nanjing Agricultural University, China.

2.3. Isolation of DNA, restriction digestions and transformation

The genomic DNAs of *K. marxianus* KM-0 and *A. niger* F4 were isolated by using a TIANamp Yeast DNA Kit (TIANGEN, Beijing, China) and used for amplification of the exo-inulinase gene and endo-inulinase gene, respectively. Purification of plasmid DNAs and extraction of DNA fragments from agarose gels were performed by using a Plasmid Miniprep Purification Kit and a Plus Gel Elution Kit (GMbiolab Co., Ltd., China), respectively. DNA restriction enzymes and DNA ligase were purchased from NEB (New England