Process Biochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Efficient digestion of chitosan using chitosanase immobilized on silica-gel for the production of multisize chitooligosaccharides

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ARTICLE INFO

Article history:
Received 19 May 2014
Received in revised form 20 August 2014
Accepted 4 September 2014
Available online xxx

Keywords:
Multisize chitooligosaccharides
Chitosanase
Immobilized enzyme
MALDI-TOF
Kinetic parameters

ABSTRACT

Chitosanase-coated silica-gels were prepared via cross-linking of the chitosanase onto silica-gels for the efficient production of multisize chitooligosaccharides (MCOs) in a continuous process. The kinetic aspects of immobilized chitosanase (IMMCTase) were investigated based on the reaction time, production of MCOs, and MALDI-TOF mass analyses to achieve maximum bioconversion of high molecular weight chitosan (HMWC) to MCOs. IMMCTase revealed a negligible loss of chitosanase activity after multi uses in continuous digestion of HMWC. The optimal temperature of IMMCTase was 37 °C, and kinetic parameters toward HMWC were determined to be K_m = 1.45 mM and V_{max} = 360 μmole/μg/min, respectively. Under optimal conditions, the recovery of enzyme activity of IMMCTase was determined to be 82.3%, thus indicating that it can still be reused few more times. In conclusion, use of IMMCTase resulted in rapid and efficient digestions of HMWC with consistent results to produce MCOs.

1. Introduction

In recent years, studies of the biological activities of chitooligosaccharides have enormously expanded its applicability to various research fields such as biomedicine [1,2], agricultural science [3,4], biofunctional food [5], and cosmetics [6,7], where chitooligosaccharides use in these industries is critical. Chitosan, unbranched linear polysaccharide of β-1,4-linkage, is widely distributed in nature and prepared in partially or fully deacetylated form of chitin, which is regarded as the second most abundant natural biopolymer after cellulose. Due to its cationic nature, chitosan, which comes in different sizes, presents a wide variety of biological and physicochemical properties including antimicrobial [8,9], antioxidant [10,11], anti-tumor [12] and anti-inflammatory effects [13,14]. Therefore identification, quantification and characterization of chitooligosaccharides are of a great importance to better understand the relationship between the size of the chitosan molecules and their biological activities [15]. Interestingly, enzyme immobilizations have been applied and successfully employed recently, using a variety of approaches, including protein digestion for applications in proteomics thus providing a highly stable, active and reusable system [16]. Although various methods with well established strategies and great efforts have been put forth to develop an effective platform to enable more research on the biological activity of multisize oligosaccharides (MCOs), sample preparation steps for the digestion of high molecular weight chitosan (HMWC) remain one of the most time consuming steps to generate MOCs. As a result, HMWC and its MCOs have attracted considerable attention in various research fields including biological, agricultural and pharmaceutical sciences. It is noteworthy that the poor solubility of HMWC in water limits its use for multi potential application in various fields considerably. Therefore, the aim of the studies has focused on the bioconversion of HMWC to MCOs, since chitooligosaccharides use is more advantageous than HMWC. To develop efficient process for the production of MCOs, various methods such as enzymatic and chemical hydrolyses have been used for several decades. The enzymatic methods appear more generally preferable than chemical methods, because the reaction is performed under gentler conditions. However, the prohibitive cost of chitosanases in addition to its random and inefficient process of deriving very low molecular weight of chitosan, limit their wide application for the production of MCOs on an industrial scale. In order to overcome these constraints in MCOs production, and achieve it with relatively lower cost, we have applied an approach to immobilize chitosanase, and characterized its function in the present study. The advantages of using chitosanase, immobilized

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http://dx.doi.org/10.1016/j.procbio.2014.09.003
1359-5113/

onto solid phase, have widely been reported [16–18], including increased biocatalyst stability and the opportunity to reuse the immobilized enzyme with concomitant reduction of process costs, as well as the feasibility of continuous operation for the bioconversion of HMWC to MCOs or glucosamine [19,20]. In this study, we characterized the biochemical properties of chitosanase immobilized onto glutaraldehyde–silica gel using HMWC, and performed enzymatic digestion systematically for the production of MCOs, prompted by the importance of chemical composition and molecular weight of MCOs in terms of reproducibility, stability and potential reusability of IMMCTCase.

2. Materials and methods

2.1. Chemicals

A high molecular weight chitosan (HMWC, 200 kDa, 95% deacetylation degree, DD) and glucosamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were as the highest grade available. Silica-gel 60 with size ranging from 0.063 to 0.2 mm was purchased from Merck (Damstardt, Germany), and PAHBAH saline (4-Hydroxy-benzhydrazid), glutaraldehyde, and (3-aminopropyl) triethoxysilane (3-APTES) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HMWC dissolved in 2% acetic acid was used as substrate. Chitosanase from *Streptomyces griseus* was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) and Iron(III)chloride for ferric reducing antioxidant power (FRAP) assay [21], and DPPH (1,1-diphenyl-2-picrylhydrazyl) for free radical scavenging activity [22] were purchased from Sigma (Sigma–Aldrich Co, St Louis, MO, USA). All other chemicals used in this study were of reagent grade.

2.2. Synthesis of silanized silica-gels

Activation of silica-gels to use as a solid matrix was performed following the modified method [23]. Briefly, one gram of silica-gels was dried at 100 °C for 2 h, cooled at room temperature, and silanized with 30 ml of acetone containing 0.5 M 3-APTES at room temperature for 12 h, washed with distilled water extensively, and dried at 50 °C for 3 h. Silanized silica-gels were added to 30 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing wide range from 0.05 to 2.0 M glutaraldehyde and incubated at room temperature for 2 h. The activated silica-gels were then washed with distilled water extensively, and dried at 50 °C for 4 h. Activated silica-gels were kept in a desiccator to prevent getting moisture.

2.3. Enzyme immobilization on activated silica-gels

Purified chitosanase (<2.0 U/2 ml) in 0.1 M sodium phosphate buffer (pH 7.0) from *S. griseus* was added to the activated silica-gels and allowed to stand for 48 h at 4 °C with stirring in occasionally. The immobilized chitosanase (IMMCTase) was washed with 0.1 M sodium phosphate buffer (pH 7.0) and then recovered by filtration. IMMCTase was then collected and stored at 4 °C until use. In addition, to elucidate the effect of monosaccharides including glucosamine on the immobilization efficiency, each sugar was added to be 0.1% into 2 ml of chitosanase solution in 0.1 M sodium phosphate buffer (pH 7.0). Mixtures were incubated at 4 °C from 2 h, after which the enzyme solutions were used for immobilization as described above.

2.4. Measuring chitosanase activity

Chitosanase activities of free enzyme and IMMCTase were determined by measuring the concentration of reducing sugars derived from the hydrolysis of HMWC spectrophotometrically. The enzyme reaction was performed at various temperatures ranging from room temperature to 60 °C to evaluate the optimum temperature, and continuous process was performed under the optimum temperature of free enzyme and IMMCCTase in time dependent manner (0–24 h). To assess the enzyme activity, 0.2 g of IMMCTase was added into 5 ml of 2% chitosan solution and same volume of 2% chitosan solution in separate tubes were prepared for both positive and negative control in the presence or absence of free chitosanase, and then incubated the tubes at 37 °C for 4 h. Samples with free chitosanase were incubated to denature the enzyme at 98 °C for 10 min and centrifuged at 13,500 rpm for 5 min to remove the denatured enzyme. In order to quench the enzyme reaction of IMMCTase, reaction solution was filtered using Whatman No. 1 paper to recover the IMMCTase and filtrates were incubated at the same condition to denature enzyme, which may possibly be released from the matrix.

2.5. Determination of the optimum temperature and stability

The optimum temperature and stability of IMMCTase were determined by a continuous process for the HMWC degradation. One unit of IMMCTase was defined as the amount of enzyme that catalyzed the conversion of μmol of reducing sugars per minute under the assay conditions. The relative activity of IMMCTase was expressed in U/g matrix unit; calculated as a percentage of the maximum activity of free enzyme. The amount of immobilized protein was calculated from the difference between total protein and the amount remaining in the solution after immobilization. The concentration of unbounded protein was determined based on Bradford method [24] using Bio-Rad protein assay reagent according to the manufacturer's protocols (Bio-Rad Laboratories, Hercules, USA). In addition, TLC analysis using supernatant was performed with 5–10 μl of each sample to elucidate the random size of the product. The mobile phase for TLC was composed of a mixture of 1:6:1:3 of ethyl acetate:1-propanol:ammonia:H_2O, respectively. After flowing of the mobile phase up in the silica gel slide, the appearance of the spots was developed using a solution consisting of 3% cupuric acetate in 8% phosphoric acid.

2.6. MALDI-TOF mass analysis

The average molecular weight (avMW) of MCOs was determined based on matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF mass) analysis (Voyager-DE TM STR Biospectrometry Workstation, Applied Biosystems Inc., NCIRF, Korea). Weighted mean based on the MW of glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) was applied for considering the relative importance of each value measured in MALDI-TOF mass to determine the avMW of MCOs.

2.7. Statistical analysis

The statistical significance of the difference between mean values in each experiment was determined by the student's *t*-test. *P*-values less than 0.01 were considered as significant values. All data were expressed as the means ± SD of activity from at least three separate experiments, unless indicated as the averages of all experiments performed in this study.

3. Results and discussion

3.1. Immobilization of chitosanase on silica-gel

The preparation of covalently attached chitosanase on silanized silica-gels was performed followed by the incubation of the

Please cite this article in press as: Song JY, et al. Efficient digestion of chitosan using chitosanase immobilized on silica-gel for the production of multisize chitooligosaccharides. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.09.003