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Investigating and optimizing the immobilization of levansucrase for increased transfructosylation activity and thermal stability

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ABSTRACT

Levansucrase (LS) represents a key enzyme in glycoside synthesis of novel prebiotics and β-2,6-levan. The study of the effects of immobilization parameters of LS, produced from *Bacillus amyloliquefaciens*, onto glyoxyl agarose-iminodiacetic acid/Cu (glyoxyl agarose-IDA/Cu) by response surface methodology revealed the significance of their interactive effects. Retention of activity was altered by interactive effects from buffer molarity/time and buffer pH/buffer molarity. The optimized immobilization conditions were identified to be a protein loading of 9.09 mg protein/g support, a buffer concentration of 608 mM at pH 6.8 and an incubation time of 49 h. Normally a reducing agent is applied to the immobilized enzyme in order to promote the formation of covalent bonds. This step was replaced with the addition of the ionic polymer polyethylenimine (PEI), which provided a better compromise between retained activity and thermal stability of the immobilized LS. Indeed, LS immobilized onto glyoxyl agarose-IDA/Cu/PEI had a retention of activity of 70.91% with a protein yield of 44.73% and an activity yield of 54.69%, while exhibiting a half-life 4.7 times higher than that of the free LS at 50 °C.

1. Introduction

Fructooligosaccharides (FOSs) constitute a class of functional ingredients, whose potential health benefits in terms of supporting intestinal health and reducing the risk of cancers are increasingly being recognized [1]. Besides acting as prebiotics [2–4], FOSs can be used as non-cariogenic sweetener replacing agent in food production [5]. On the other hand, β-(2,6)-levan polysaccharides have shown antitumor and antidiabetic activities in addition to their stabilizing, formulation aid, encapsulating agent and flavour carrier capacities relevant to the pharmaceutical, cosmetic and chemical industries [6–9]. Levansucrase (EC 2.4.1.10, LS) has been studied by our group and others as a potential biocatalyst for synthesizing FOSs and β-(2,6)-levan polysaccharides [10–16]. Belonging to glycoside hydrolase family 68, LS contains a five-fold, β-propeller typology [17]. The LS active site contains subsites, which orient and stabilize the fructosyl and glucosyl residues of sucrose as it enters the LS active site [18]. The amino acid composition of the subsites of LSs defines their substrate affinity and the predominant reactions, exchange, hydrolysis, oligomerization and polymerization [18,19]. LSs from different microbial sources differ with respect to their reaction selectivity (hydrolysis/transfructosylation) and oligo-/polymerization ratio [19]. Recently, some hypotheses and structural features have been put forward to describe the reaction selectivity and the polymerizing activity of LSs [18,20].

Few studies [21–23], including our own [15,16], revealed that modulating LS's macro/microenvironments may afford means for favoring its reaction selectivity toward transfructosylation. For instance, immobilization may help modulate the hydrophilic/hydrophobic balance of LS microenvironment and hence optimize the reaction selectivity. In addition, immobilization of enzymes can allow for the easy reuse of the biocatalyst and can promote enzyme stabilization [24]. Site-directed immobilization orients the enzyme on the support by having immobilization occur through specific regions on the enzyme. Modifications of solid supports can limit immobilization to where there is the highest density of reactive residues on the enzyme [25]. Multipoint covalent attachments via short

spacer arms can also increase the stability of an enzyme by reducing flexibility. Immobilization parameters may also affect not only the enzyme orientation, but also the immobilization rate and the homogeneous distribution of enzyme on the supports [26,27].

Previous immobilization of LS had been performed using various supports including hydroxyapatite, titanium-activated magnetite, chitin beads, chitosan modified with glutaraldehyde as well as the production of CLEAs [26,28–32]; yet no study performed a through optimization of

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the immobilization conditions of LS. In our previous studies, glyoxyl-agarose-IDA/Cu support was identified as the most appropriate support for the immobilization of LS from *Bacillus amyloliquefaciens*. This support was identified upon a screening performed using modified and unmodified Eupergit® C, Sepabeads® and glyoxyl agarose supports as well as unmodified Sepabeads® HA [16]. However, compromising between the retained LS activity upon immobilization on glyoxyl agarose-IDA/Cu support, the stability and the reaction selectivity needs to be addressed. The present study was aimed at the investigation and the understanding of the synergism between the immobilization parameters for an efficient modulation, at the molecular level, of the properties of LS immobilized on glyoxyl agarose-IDA/Cu support. In this regards, the effects of protein loading, immobilization buffer concentration, pH and immobilization time on the retention of LS activity, immobilization yield, activity yield, and transfructosylating/hydrolytic activity ratio were investigated using response surface methodology (RSM). RSM allows the development of mathematical models to assess the statistical significance of the variables being studied and their combined effects upon the system as a whole [33]. Understanding of the interactive effects of immobilization parameters are expected to allow the optimization of the retained LS, but also an improved modulation of its reaction selectivity. The use of sodium borohydride to reduce Schiff bases and stabilize LS immobilized on heterofunctional glyoxyl agarose-IDA/Cu had caused a decreased in the retained LS activity. Alternative stabilization of the immobilized LS by crosslinking with glutaraldehyde or through interactions with polyethylenimine (PEI), which does not require the use of a reducing agent, was successfully examined and detailed in this study.

2. Materials and methods

2.1. Materials

Sucrose, D-(−)-fructose, D-(+)-glucose, 3,5-dinitrosalicylic acid, NaOH, polyethylene glycol (PEG) 200, potassium sodium tartrate ($KNaC_4H_4O_6$), $NaIO_4$, $NaBH_4$, iminodiacetic acid ($HN(CH_2CO_2H)_2$), glutaraldehyde ($OHC(CH_2)_3CHO$), polyethylenimine ($H(NHCH_2CH_2)_nNH_2$) and $CuSO_4$ were obtained from Sigma Chemical Co. (St. Louis, MO). $CaHPO_4$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}7H_2O$, $Na_2HPO_4{\cdot}2H_2O$, $NaMoO_4{\cdot}2H_2O$, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $NaHCO_3$, glycerol, bovine serum albumin (BSA) and yeast extract were obtained from Fisher Scientific (Fair Lawn, NJ). Agarose 10BCL was purchased from Agarose Bead Technologies. Low molecular weight levan was produced by the procedure described by Tian et al. [12]. Orafti-P95 was provided by Beneo (Morris Plains, NJ) *B. amyloliquefaciens* (ATCC 23350) was obtained from American type culture collection (Manassas, VA, USA). Bradford reagent concentrate was provided by Bio-Rad (Missasauga, ON, Canada).

2.2. Production of levansucrase from Bacillus amyloliquefaciens

Production of LS from *B. amyloliquefaciens,* strain ATCC 23350, followed the protocol described by Tian et al. [10] *B. amyloliquefaciens*, pre-cultured aerobically in nutrient broth (8 g/L), was transferred (4 mL) to a 1-L baffled Erlenmeyer flask, which contained 400 mL of modified mineral salt medium consisting of (g/L) $Na_2HPO_4{\cdot}2H_2O$ (2.67), KH_2PO_4 (1.36), $(NH_4)_2SO_4$ (0.5), $FeSO_4{\cdot}7H_2O$ (0.005), $MnSO_4{\cdot}H_2O$ (0.0018), $NaMoO_4{\cdot}2H_2O$ (0.0025), $CaPO_4{\cdot}2H_2O$ (0.01), $MgSO_4{\cdot}7H_2O$ (0.2) and yeast extract (10). Sucrose was used to induce the production of LS. The media was incubated at 35 °C at 150 rpm for 11 h, afterwards the solution was centrifuged (8000 rpm, 20 min) to retrieve the pellet. Potassium phosphate buffer (50 mM, 37.5 mL), pH 6 containing 1% Triton X-100 was used to resuspend the bacteria cells. Ultrasonification was applied to the cells for 6 min and 25 s, set at 15 kHz with 25/50 s cycles. The cellular debris was separated by centrifugation (8000 rpm, 4 °C) for 15 min after which PEG 200 (30% v/v) was added to the supernatant to partially purify LS. The solution was slowly stirred at 4 °C for 14 h. The solution was centrifuged (12,000 rpm, 4 °C) for 45 min, the supernatant was removed, and the pellet was resuspended in a minimum volume of potassium phosphate buffer (50 mM) pH 6. The resuspended pellet was dialysed against potassium phosphate buffer (5 mM, 30 L) with a molecular weight cut-off of 6–8 kDa, then lyophilized until dry. The protein content of the powder was determined using the Bradford protein assay, using bovine serum albumin as a standard [34].

2.3. LS activity assays

The total, transfructosylating and hydrolytic activities of LS were assessed. The total LS activity is expressed as the μmol of reducing sugars released per min per mL of enzyme. LS (250 μL) in potassium phosphate buffer (50 mM) pH 6, was added to sucrose solution (1.8 M, 250 μL), and the reaction mixture was incubated at 30 °C for 20 min 3, 5-Dinitrosalicyclic acid (DNS) assay was used to measure the total reducing sugars [35]. The hydrolytic activity was estimated as the μmol of free fructose released per min per mL of enzyme. The transfructosylating activity was calculated as the μmol of fructose transferred to an acceptor molecule per min per mL of enzyme. This was measured by subtracting the amount of glucose from the amount of free fructose. The monosaccharides were quantified by high-pressure-anionic-exchange-chromatography with a pulsed amperometric detector (HPAEC-PAD, Dionex) using a CarboPac PA-20 (3 × 150 mm) column and analysed using Chromeleon Software. The products were separated using isocratic elution with 10 mM NaOH at a flow rate of 0.5 mL/min and maintained at 32 °C.

2.4. Functionalization of glyoxyl agarose-IDA/Cu

The glyoxyl-based supports were prepared following the protocol of Mateo et al. [25].

Epoxy-activated agarose

$NaBH_4$ (2 g) was added to NaOH (0.656 M, 440 mL) and acetone (160 mL), which was kept on ice. Agarose 10-BCL (100 g) and epichlorohydrin (110 mL) were added and the mixture was stirred 14 h at 25 °C. The support was washed with deionized H_2O (10 vols).

Glyoxyl agarose-IDA/Cu

An IDA solution (0.5 M) adjusted to pH 11, was mixed with wet, epoxy-activated agarose (10% w/v). The reaction was mixed for 36 h at 25 °C. The support was then filtered on a sintered glass filter and washed with deionized H_2O (10 vol). $NaIO_4$ (0.01 M, 5% v/v support:solution) was mixed with the support for 90 min and then washed with deionized H_2O (10 vol). The support was then mixed with a solution of $CuSO_4$ (30 mg/mL) for 1 h at room temperature. The support was filtered and washed with deionized H_2O (10 vol).

2.5. Immobilization of levansucrase

LS (9.09 mg protein/g wet support) solution was added to glyoxyl agarose-IDA/Cu support in potassium phosphate buffer (608 mM, pH 6.8). The mixture was gently mixed at 4 °C for 49 h. The LS activity and the protein content of the recovered supernatant were measured. A sodium bicarbonate buffer (608 mM, 1 mL) at pH 10 containing 20% (v/v) glycerol was added to immobilized LS, and the resulted suspension was incubated for 3 h at 4 °C to promote the formation of covalent bonds. To reduce the Schiff bases, $NaBH_4$ (1 mg/mL) was added to the high pH suspension and gently mixed at 4 °C for 30 min. Afterwards, the supernatant was recovered and tested for protein content. The support was washed with 10 vol of potassium phosphate buffer (50 mM, 1 mL), at pH 6.

The immobilized LS was resuspended in potassium phosphate buffer