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A simple method for obtaining reusable reactors containing immobilized trehalase: Characterization of a crude trehalase preparation immobilized on chitin particles

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Abstract

We propose a simple method for obtaining a stable, specific and reusable immobilized trehalase reactor for specific quantification of trehalose. Periplasmic trehalase was extracted from transformed *Escherichia coli* cells (strain Mph2 carrying the plasmid pTRE11 which harbored the trehalase gene TreA+) by osmotic shock. Periplasmic protein molecules, obtained by osmotic shock, were immobilized on chitin particles (Tyler 35) without any further step of purification. The maximal percentage of trehalase activity retained on chitin was 86% when 2.48 U of trehalase were immobilized on 0.1 g chitin. At pH 5.5 (pH optimum) the optimal temperature was 50 °C. For immobilized trehalase, the apparent Michaelis constant (K_m app.) was 0.5 mM trehalose at pH 5.5 and 30 °C with a V_m app. of 0.036 μmol of glucose min^{-1}. On the other hand, for soluble trehalase K_m was 1.32 mM at pH 5.5 and 30 °C, with a V_m of 0.011 μmol of glucose min^{-1}. The reactors stored in 50 mM sodium maleate buffer, pH 6.0, at 10 °C, for 55 days and reused 10 times, had no significant loss of activity. Furthermore, the stability of the immobilized conjugate was also tested in a columnar reactor showing no loss of activity during 40 h of continuous operation, at pH 6.0 and 30 °C.

Keywords: Trehalase; Immobilization; Trehalose quantification; Chitin

1. Introduction

Reports on chemical immobilization of proteins and enzymes first appeared in the 1960s. Since then, immobilized enzymes have been widely used in the processing of a variety of products, as well as in specific quantification of molecules. They have been increasingly used in the field of medicine [1] and more recently in homogeneous biocatalysis in organic solvents and water–organic mixtures [2]. Furthermore, immobilization has been used in the generation of peptide and protein microarrays [3] as well as in the emerging field of nanotube-biotechnology [4]. The advantages of immobilized over soluble enzymes arise from their enhanced stability and ease of separation from the reaction media, leading to significant savings in enzyme consumption. Immobilization methods range from binding to prefabricated carrier materials to packaging in enzyme crystals or powders [5].

Trehalase (EC 3.21.28) is the enzyme that specifically hydrolyses the disaccharide trehalose into two α-D-glucose molecules. It is worth mentioning that trehalose (α-D-glucopyranoside) is a potent protector of proteins, enzymes and membranes [6–9], as well as cells [10,11] and organs for transplant [12] with vast applicability in biotechnology [13]. It has been shown to protect various kinds of biomolecules

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under conditions such as desiccation, freezing, high pressure and high temperatures [14,15].

The aim of this work was to develop a simple method for producing a reusable enzymatic reactor for specific quantification of the disaccharide trehalose. It is important to emphasize that we have constructed a stable reusable reactor containing a crude enzyme preparation immobilized on chitin particles. We had previously reported [16] the immobilization of purified trehalase on aminopropyl glass for analytical use. In the present paper, we show that it was not necessary to purify *Escherichia coli* periplasmic trehalase for producing a specific enzymatic reactor for quantifying trehalose. The novelty of this procedure is that the steps for trehalase purification, such as ammonium sulfate precipitation, chromatography on Sephadex G-25 and on DEAE-cellulose, as well as the step of lyophilization were skipped. Therefore, the present approach is simpler than the previous one and also cheaper, since we have used chitin as support for enzyme immobilization instead of aminopropyl glass. Chitin is a hydrophilic, inert, non-biodegradable and highly resistant to environmental changes. It was obtained from crab carapace waste of food industry. After demineralization and deproteinization it was activated for enzyme immobilization as suggested by [17].

Trehalase was extracted from transformed *E. coli* cells (strain Mph2 carrying the plasmid pTRE11 which harbored the trehalase gene TreA+). This system provided a 58 kDa trehalase, which could be easily released from the periplasmic space by osmotic shock [18]. Protein molecules present in the fluid, obtained by osmotic shock, were immobilized on chitin particles (Tyler 35) without any further step of purification, leading to a specific reusable enzymatic microreactor for routine trehalose quantification. It is important to mention that the only glycosidase activity detected in the osmotic shock fluid (OSF) was that of trehalase. Therefore, immobilized OSF proteins could specifically quantify trehalose in biological samples, which contained other types of carbohydrates.

2. Materials and methods

2.1. Trehalose

D(+)trehalose, obtained from Sigma Chemical Co, was used in the majority of the experiments. Trehalose from biological samples (*S. cerevisiae*, 12 mg dry weight) was also tested as substrate for trehalase. For this purpose trehalose was extracted with either water [19] or ethanol [20].

2.2. Microorganism preparation

E. coli Mph2 (*pho* A, *tre* A^-) carrying pTRE11 was used for trehalase extraction. The trehalase gene was inserted into a portion containing the tetracycline resistance gene. The strain and the multicopy plasmid (which also carried the ampicillin resistance gene) were a kind gift from Peter Postma, University of Amsterdam, The Netherlands. The transformation was performed according to the procedure of [21]. The basic growth medium for obtaining cells, for further trehalase extraction, consisted of 1% bactotryptone, 1% NaCl, 0.5% yeast extract and 50 μg/mL ampicillin, pH 7.5. Cells were grown in 2-L flasks containing 400 mL of medium at 37 °C on a shaker at 250 rpm. The non-transformed Mph2 strain was used as control. Cell density was monitored by turbidity measurements at 590 nm.

2.3. Extraction of periplasmic trehalase

E. coli cells, harvested in stationary phase, were submitted to osmotic shock, as described by [22]. The osmotic shock fluid containing extruded trehalase was the material used for direct immobilization on chitin particles. The transformed cells harbored a multicopy plasmid containing a cloned trehalase gene, which was over-expressed and codified for a periplasmic trehalase. Trehalase was the major protein of the periplasmic space and could be released by osmotic shock. The osmotic shock fluids tested in this work had 1.3–5.3 U of trehalase/mL, 0.22–6.2 mg of protein/ml and average specific trehalase activity of 8.25 U/mg of protein.

2.4. Chitin preparation

Chitin was obtained from crab carapace, waste of food industry. Chitin particles (Tyler 35) were prepared as suggested by [17]. After deproteinization and demineralization, chitin was activated with hexamethylenediamine (HEMDA) and glutaraldehyde for further enzyme immobilization. One hundred milligrams of chitin particles were activated with 2% (w/v) HEMDA at 40 °C for 2 h. After this reaction the excess of HEMDA was removed with a Pasteur pipette, and then 3 mL of 3% (v/v) glutaraldehyde were added to the preparation. After 30 min at 30 °C HEMDA-glutaraldehyde treated chitin particles were washed five times with 500 μL absolute ethanol for removing glutaraldehyde molecules. Subsequently, the material was washed five times with 50 mM maleate buffer, pH 6.0.

2.5. Trehalase immobilization on chitin

After the osmotic shock, the extruded fluid was collected and then added to 250 mg of active chitin. Excess of OSF was added in order to guarantee saturation of the support. Incubation time, optimal pH and optimal temperature for immobilization reaction were investigated. After the appropriate reaction time the supernatant was carefully collected. Then, the trehalase-chitin particles were washed three times with 50 mM sodium maleate pH 6.0; three times with 1 M NaCl; three times with 0.5 M $NaHCO_3$; and, again, three times with 50 mM sodium maleate pH 6.0 to remove unbound enzyme molecules. A sample of trehalase-chitin (TC) was dried on filter paper at room temperature and weighed. Approximately