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Development of a continuous solid phase process for reduction and thiol-dependent immobilization of yeast β-galactosidase

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ABSTRACT

Reduction and covalent immobilization of *Kluyveromyces lactis* β-galactosidase through disulfide bonds onto thiolsulfinate-agarose was performed using two fixed-bed mini-reactors connected in series, one packed with thiopropyl-agarose (a solid phase reducing agent) and the other with thiolsulfinate-agarose (a thiol-reactive support). With the aim of optimizing the whole process, two reactor systems were assessed. In System I, the percolate from thiopropyl-agarose containing the reduced enzyme was re-circulated through the thiolsulfinate-agarose reactor alone. In System II, re-circulation was performed through both the reactors, improving the immobilization yield from 17% (System I) to 42% and the expressed activity from 25% (System I) to 56%. When the bio-reactor achieved with System II was fed with skimmed milk at 22 °C at a flow rate of 48 ml/h, steady state lactose hydrolysis reached 80%. In addition, it could be reused four times without losing its lactose hydrolysis capacity.

1. Introduction

β-Galactosidase (EC 3.2.1.23), commonly known as lactase, catalyzes (among other reactions) the hydrolysis of lactose producing an isomolecular mixture of glucose and galactose. Lactose is the major component of milk, whey and permeate solids, but probably the least valuable and the most difficult to utilize. Additionally it causes nutritional (malabsortion) and pollution problems (due to the high biological oxygen demand of lactose streams) [1]. In current commercial practice, the *Kluyveromyces* yeasts are the preferred source of neutral-pH lactase because of their GRAS status [2]. *Kluyveromyces lactis* β-galactosidase has a wide field of applications (not only for lactose hydrolysis but also for synthetic purposes, due to its transglycosidase activity). However, its use is limited because of economic considerations, unless it is used in an immobilized form, allowing for the reuse of the enzymatic bed. Our experience has shown that covalent reversible immobilization of enzymes is an excellent option for developing insoluble biocatalysts, giving high immobilization yields, under mild conditions and via stable covalent bonds [3–6]. The immobilization process involves the formation of disulfide bonds between exposed thiol groups on the enzyme and thiol-reactive structures (thiolsulfinate or thiolsulfonate moieties) on the support (Fig. 1).

Although *K. lactis* β-galactosidase has no superficial thiol groups (so that no covalent reversible immobilization could be achieved in its native form), a previous reduction step allowed its immobilization onto thiolsulfinate or thiolsulfonate supports [5]. One of the most convenient ways of generating sulfhydryl groups is by reduction of native disulfides with thiol-containing compounds.

We have previously reported the advantages of using solid phase reducing agents for performing this reduction process [7,8], allowing the same performance as dithiothreitol (DTT), under mild conditions and requiring less μmol of SH groups from the reducing agent per mg of protein.

Solid phase reducing agents are also an excellent option for reducing proteins before a covalent immobilization process onto thiol-reactive supports. If a soluble reducing agent such as DTT is used, its excess competes with the reduced protein for reactive groups on the support and it has to be removed. This removal is usually performed by gel filtration, but by using solid phase reducing agents it is possible to substantially cut down the time required for the overall process, since they can be easily separated by filtration [6]. Moreover, solid phase reducing agents have other advantages over soluble ones: they do not liberate any contaminating by-products, and it is possible to reuse them many times [7]. The reduction process involves the formation of an insoluble mixed disulfide followed by nucleophilic attack by a neighboring thiopropyl group on the mixed disulfide, forming a new disulfide bond with the simultaneous release of the reduced protein (Fig. 2).

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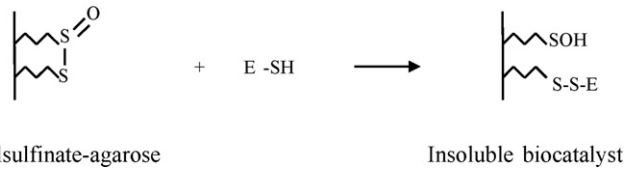

Fig. 1. Enzyme immobilization on thiolsulfinate-agarose.

In order to simplify the covalent immobilization process of *K. lactis* β-galactosidase (avoiding intermediate steps) and to demonstrate the capacity of solid phase reducing agents for continuous enzyme reduction, we developed a continuous solid phase process (a tandem procedure), in which a solid phase reducing agent (thiopropyl-agarose) packed in a column was connected in series with another column containing a thiol-reactive support (thiolsulfinate-agarose). Enzyme reduction was performed in the first mini-reactor, while the immobilization process occurred in the second. Two mini-reactor arrangements were assessed: System I, in which the re-circulation only involved the thiol-reactive column, and System II, in which the percolate was re-circulated through both columns. We also studied the capacity of the resulting insoluble derivative for continuous lactose hydrolysis.

2. Materials and methods

2.1. Materials

Sepharose-4B and PD-10 columns (Sephadex G-25) were from Pharmacia Biotech AB (Uppsala, Sweden); *o*-nitrophenyl-β-D-galactopyranoside (ONPG), DTT, 2,2′-dipyridyl disulfide (2-PDS) and 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) were from SIGMA (St. Louis, MO). Magnesium monoperoxyphthalate (MPP) was from Merck (Damstadt, Germany); Bicinchoninic acid (BCA) Protein Assay Kit was purchased from Pierce (Rockford, IL). β-Galactosidase (β-galactoside galactohydrolase; EC 3.2.1.23) from *K. lactis* (Maxilact LX-5000) was a gift from Gist Brocades (Cedex, France). The glucose determination kit was purchased from REACUR S.A. (Montevideo, Uruguay). All other products used were of reagent or analytical grade.

2.2. Synthesis of thiopropyl-agarose

Thiopropyl-agarose with a content of 39 μmol thiopropyl groups per ml was prepared essentially as reported by Axen [9].

2.3. Titration of thiol groups

Thiol content of thiopropyl-supports and reduced enzyme was determined spectrophotometrically at 343 nm by titration with 2-PDS (saturated solution, 1.5 mM) in 0.1 M sodium phosphate, pH 8.0 [10]. Quantitative determination of sulfhydryl groups in solution was also performed with DTNB [11].

2.4. Preparation of thiolsulfinate-agarose (TSI-agarose)

Thiolsulfinate-agarose with a content of 15 μmol thiolsulfinate groups per ml was prepared essentially as reported by Batista-Viera [12].

2.5. Protein determination

Protein content of the enzyme solutions was determined with BCA assay [13]. Immobilized protein was estimated as the difference between the amount of protein added to the gel and that recovered in the pooled supernatant and washing fractions. Bovine serum albumin was used as standard.

2.6. Activity determinations

β-Galactosidase activity in solution was assayed at room temperature using 14 mM ONPG as substrate in 20 mM potassium phosphate buffer, pH 7.0, containing 2.0 mM $MgCl_2$ and 0.1 M KCl (activity buffer); released *o*-nitrophenol was determined by measurement of absorption at 405 nm [14]. Immobilized enzyme activity was assayed by incubating 100 μl aliquots of gel suspensions (containing 10 mg of suction-dried gel derivatives) with 3 ml of 28 mM ONPG in activity buffer, using a 1-cm path length cuvette provided with magnetic stirring and absorption was measured at 405 nm. One unit of enzyme activity (EU) was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of ONPG per minute under the specified conditions.

2.7. Column procedure for enzyme reduction with thiopropyl-agarose

(i) Thiopropyl-agarose columns (3.0 ml packed gel) equilibrated in 50 mM potassium bicarbonate pH 8.5, 3 mM $MgCl_2$ (reduction buffer) were fed with 1.0, 2.0, 3.0, 4.0 and 5.0 ml of native enzyme (640 EU per ml, 8.0 mg protein/ml), respectively, at a flow rate of 2.0 ml/min. The percolate was collected and the SH content and activity were determined as described in Sections 2.3 and 2.6, respectively.

(ii) Thiopropyl-agarose columns (3.0 and 9.0 ml packed gel) equilibrated in reduction buffer, were fed with 3.0 ml of native enzyme (640 EU per ml), at a flow rate of 2.0 ml/min. The percolate was collected and the SH content and activity were determined as described in Sections 2.3 and 2.6, respectively.

2.8. Column procedure for enzyme immobilization on TSI-agarose

(i) Aliquots (7.5 ml) of the percolate collected after enzyme reduction with a thiopropyl-agarose column (9.0 ml packed gel), containing 100 EU/ml and 1.5 mg protein/ml, were re-circulated at 22 °C with a flow rate of 1.0 ml/min through a TSI-gel column (3.0 ml packed gel) equilibrated in reduction buffer. Aliquots of

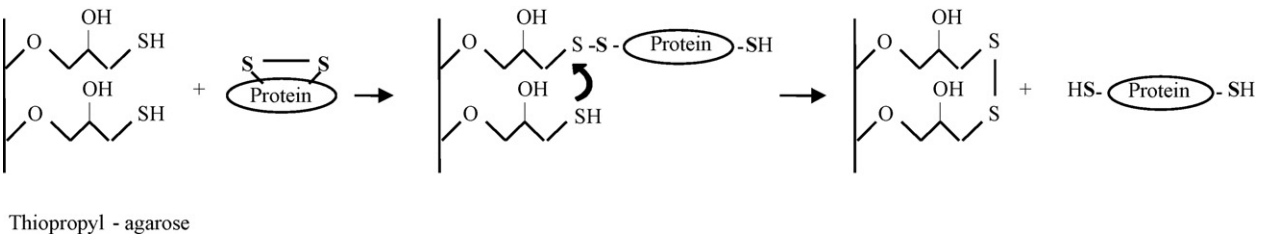

Fig. 2. Reduction of disulfide bonds in proteins with thiopropyl-agarose.