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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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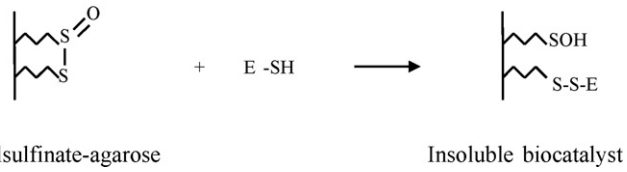


Fig. 1. Enzyme immobilization on thiolsulfinate-agarose.

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

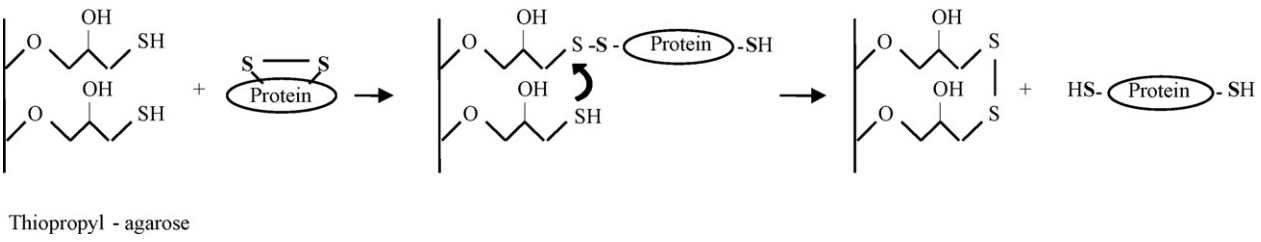


Fig. 2. Reduction of disulfide bonds in proteins with thiopropyl-agarose.

the re-circulated percolate were taken at different times (1, 3, 4 and 16 h) and β-galactosidase activity was measured.

(ii) The same procedure was followed as in (i) but re-circulating for 16 h at 4 °C or 22 °C with a flow rate of 1.0 ml/min through TSI-gel columns (3.0 and 9.0 ml packed gel) equilibrated in reduction buffer. The effect of the re-circulation process on reduced enzyme stability was also assessed by re-circulating the enzyme for 16 h at 4 °C or 22 °C in the absence of gel.

2.9. Tandem procedure for enzyme reduction and immobilization

2.9.1. System I

Native enzyme (3.0 ml, containing 8.5 mg protein/ml, 640 EU/ml), was pumped through the reducing mini-reactor (a column with 9.0 ml packed thiopropyl-agarose, equilibrated in 50 mM potassium bicarbonate buffer pH 8.5, 3 mM $MgCl_2$) at a flow rate of 2.0 ml/min. The resulting reduced enzyme fed the second reactor (a TSI-column, 9.0 ml of packed gel). Then the percolate was re-circulated for 16 h at 22 °C through the thiolsulfinate-agarose reactor (Scheme 1A). Finally this mini-bio-reactor was sequentially washed with reduction buffer, with and without 0.5 M NaCl (in order to elute non-specifically bound proteins) and equilibrated in activity buffer. Activity was determined in the re-circulated percolate, the washes and on the washed bio-reactor.

2.9.2. System II

The same procedure followed for System I, (see Section 2.9.1), except that in this case, re-circulation was performed through both mini-reactors (Scheme 1B). After this operation, both mini-reactors were disconnected and the residual thiol content on the solid phase reducing agent was measured.

2.10. Lactose hydrolysis

(i) The bio-reactor achieved with System II was fed continuously with skimmed milk at 22 °C at a flow rate of 48 ml/h. The degree of hydrolysis was determined by monitoring the release of glucose using an enzymatic method (glucose oxidase combined with peroxidase).

(ii) *Reuse*: after performing the lactolysis process for 24 h (first use), the amount of glucose formed was determined and the column was washed with activity buffer. Then the washed column was used for a second time in the same way as the first time. This protocol was carried out four times (between each, the column was washed).

The initial content of glucose in milk was zero.

3. Results and discussion

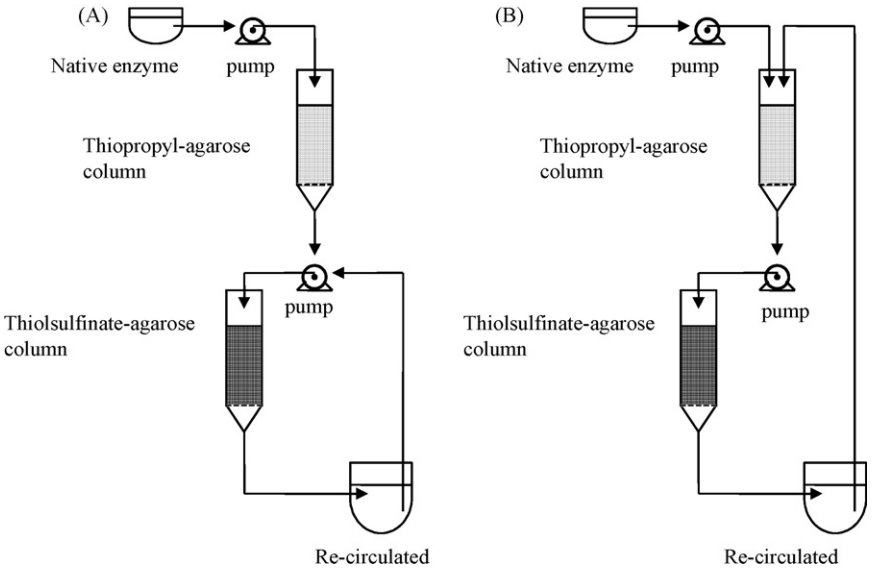
3.1. Reduction and immobilization of β-galactosidase

Neutral lactases have been immobilized by different methods and on several supports, but in most of the cases with low yields and through irreversible bonds [15]. To overcome these drawbacks, covalent reversible immobilization is an excellent option for immobilizing enzymes, since this method is performing under mild conditions, through stable reversible bonds and allowing the reuse of the support (reducing the cost of the global process).

The β-galactosidase from *K. lactis* was selected for this study because it has a wide range of biotechnological applications based on its hydrolytic and synthetic functions. Although it is possible to detect a low level of free SH groups in the enzyme by titration with thiol reagents, they do not react with thiol-reactive supports and no covalent immobilization of the enzyme can be achieved in its native form. The reduction of native disulfides has proved to be an alternative for increasing the SH content of the enzyme (without affecting its specific activity) allowing its covalent immobilization [5].

The main purpose of our approach was to develop a continuous solid phase process that combines the benefits of using solid phase reducing agents (like thiopropyl-agarose) with the advantages of covalent reversible immobilization (using thiolsulfinate-agarose as a thiol-reactive support).

In spite of the fact that agarose is not suitable for most large-scale applications, we chose agarose-based supports in order to work out the basis of the continuous process. This decision was based on our previous experience on working with other supports, such as acrylic resins (TSK-gel Toyopearl HW-65 F and Eupergit C) both for immobilization [5] and for solid phase reducing treatment [8]. We found that interactions between proteins and acrylic resins were more complex than with agarose. Using Toyopearl-based supports,



Scheme 1. Tandem procedures for β-galactosidase reduction and immobilization: (A) System I; (B) System II.

Table 1
Incidence of thiopropyl-agarose bed size on the reduction of *K. lactis* β-galactosidase

Enzyme	Reductive reactor: packed gel (ml)	μmol SH/mg protein[a]	$\mu mol\ SH_{reduced\ enzyme}/\mu mol\ SH_{native\ enzyme}$
Native	–	0.012 ± 0.001	–
Reduced	3	0.022 ± 0.003	1.8
Reduced	9	0.033 ± 0.003	2.8

The enzyme load applied was 24.0 mg protein (1920 EU) in 50 mM potassium bicarbonate pH 8.5, 3 mM $MgCl_2$.

[a] Results represent averages of at least four experiments.

the expressed lactase activity was strongly salt dependent and the reduction process was affected by the smaller hydrophilicity of the resins, giving solid phase reducing agents with more compact structures in aqueous milieu, affecting the spatial distribution of their thiol groups.

The first stage of this research involved the optimization of the reduction and immobilization processes, separately. We began by studying the effect of the enzyme load on the reduction process, using a column with 3.0-ml packed thiopropyl-agarose. Independently of the applied load (8–40 mg protein), the reduction degree was constant (data not shown). Only a twofold increase in the enzyme's initial SH content was obtained, suggesting that applied loads exceeded the reducing capacity of the column. In order to verify that this was the case, the bed size of the mini-reactor was increased from 3.0 to 9.0 ml of packed gel. As expected, higher amounts of thiopropyl-agarose was correlated with an increase in the extent of reduction, achieving the same reduction degree as working in solution with DTT or batchwise with thiopropyl agarose [7] (Table 1).

Based on our previous knowledge about the kinetics of protein reduction with solid phase reducing agents batchwise [7], relatively low flow rates were used during column procedures, thus increasing the residence time and allowing more extensive interactions between protein disulfides and the thiol groups of the thiopropyl-agarose. During the reduction process, a total of 17 ml of percolate volume (100 EU/ml, 1.5 mg protein/ml) was collected. When aliquots of this reduced enzyme were applied to a TSI-agarose column, the resulting immobilization yields were found to be strongly dependent on re-circulation time. After the first 4 h, the immobilization yield was nearly 10%, and almost 90% of the applied enzyme was recovered (with unchanged activity) in the re-circulated percolate (Fig. 3).

This result was in agreement with the fact that the covalent reversible immobilization of *K. lactis* β-galactosidase is a slow process [5], maximum immobilization yields being achieved after batchwise incubation overnight (16 h). To work out optimal conditions for immobilization of the reduced enzyme, the percolate was re-circulated for 16 h under different conditions of temperature and bed size (Table 2).

The optimal conditions for column immobilization on TSI-agarose required re-circulation of the percolate through a column bed between 3.0 and 9.0 ml for 16 h at 22 °C, allowing the highest immobilization yield and coupling efficiency (28% and 41%, respectively).

These results are consistent with the strong dependence on pH of the immobilization process of a thiol-protein onto thiol-reactive supports, and with the fact that thiol groups generated by reduction are unstable at pH ≥ 7.0 [7]. Since the best pH for enzyme reduction was 8.5 [5], and we were attempting to design a continuous process, the immobilization had to be performed at this pH. Therefore, the exposed SH groups on the reduced enzyme were more easily oxidized during the immobilization process at pH 8.5, this meant there were less interactions between enzyme's SH groups and the reactive groups of the support. This in turn affected the immobilization yield, which was lower than that achieved in a batchwise procedure at pH 7.0 [5].

An increase in the bed size of TSI-agarose column from 3.0 to 9.0 ml packed gel had no significant influence on the immobilization yield (Table 2). However, as the immobilization yield usually decreases with the amount of applied protein, we selected a 9.0-ml bed column of thiol-reactive support for developing the tandem systems. Because a continuous process was to be performed, we had to apply the whole percolate volume from the thiopropyl-column to the TSI-column, equivalent to 25.5 mg of protein.

The re-circulation process dramatically affected enzyme stability, and this effect increased as the temperature was raised from 4 to 22 °C; thus, after 16 h, the reduced enzyme lost 13% and 64% of its initial activity, respectively (control experiments without gel). These results were relevant because they demonstrated that the immobilization process stabilized the enzyme, especially at 22 °C, since in every immobilization experiment performed the recovered activity (total of enzyme units expressed on the gel and recovered in the re-circulated percolate) was more than 60% of the applied activity (Fig. 4).

Although thermal stability of the reduced enzyme was higher at 4 °C, we decided to perform the continuous solid phase process at 22 °C since higher figures for immobilization and coupling efficiency yields were achieved at this temperature (Table 2).

3.2. Tandem procedures for enzyme reduction and immobilization

Encouraged by the reduction and covalent immobilization results achieved separately, we attempted in a second stage the assessment of two tandem procedures for the consecutive reduction and immobilization of β-galactosidase from *K. lactis*. In System

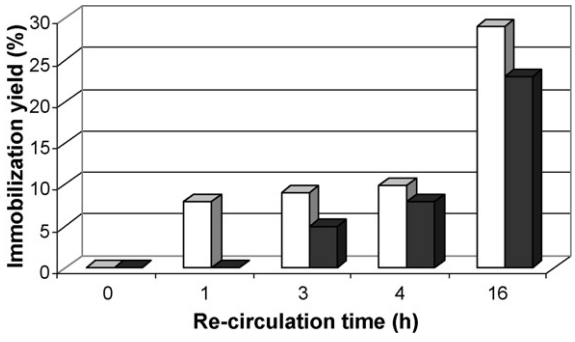


Fig. 3. Kinetics of reduced enzyme immobilization on TSI-agarose, under re-circulation at: (□) 22 °C and (■) 4 °C.

Table 2
Immobilization of reduced enzyme onto TSI-agarose column under different conditions

Column bed (ml packed gel)	Temperature (°C)	Immobilization yield[a,c] (%)	Coupling efficiency[b,c] (%)
3	22	24 ± 1	38 ± 1
3	4	13 ± 1	24 ± 1
9	22	28 ± 1	41 ± 1
9	4	23 ± 1	27 ± 1

Aliquots (7.5 ml, specific activity 67 EU/mg) of the percolate volume collected after enzyme reduction with thiopropyl-agarose columns, were re-circulated for 16 h through TSI-columns containing 3.0 or 9.0-ml packed gel, at 4 or 22 °C, respectively.

[a] Defined as: $(EU_{expressed\ on\ the\ gel}) \times 100/(EU_{applied})$.

[b] Defined as: $(EU_{expressed\ on\ the\ gel}) \times 100/(EU_{applied} - EU_{recovered\ in\ the\ re\text{-}circulated\ percolate+washes})$.

[c] Results represent averages of at least four experiments.

Table 3
Immobilization performance using tandem Systems I and II

Tandem systems	$EU_{applied}$	$EU_{recovered}$ [a]	$EU_{expressed\ on\ the\ gel}$	Immobilization yield[b] (%)	Coupling efficiency[c] (%)
I	1700	541	297	17	25
II	1700	431	713	42	56

[a] $EU_{washes} + EU_{in\ the\ re\text{-}circulated\ percolate\ after\ 16\,h}$.
[b] Defined as: $(EU_{expressed\ on\ the\ gel}) \times 100/(EU_{applied})$.
[c] Defined as: $(EU_{expressed\ on\ the\ gel}) \times 100/(EU_{applied} - EU_{recovered\ in\ the\ re\text{-}circulated\ percolate+washes})$.

I the percolate from the thiopropyl-agarose containing the reduced enzyme, was re-circulated only through the thiol-reactive reactor (TSI-agarose), while in System II it was re-circulated through both the TSI-agarose and reducing reactors (Scheme 1).

Immobilization yields and coupling efficiencies were strongly dependent on the way the re-circulation was performed, since the results using System II were twice as high as those using System I (Table 3).

Although System I was similar to a classical column immobilization procedure (see Section 2.8), its immobilization yield and coupling efficiency were lower than those achieved in a classic immobilization due to the high level of applied protein and the SH oxidation that occurred during re-circulation. Such oxidation might principally involve superficial thiol groups on the protein, which are very essential for the immobilization process since they can react with the thiol-reactive groups on the TSI-column with little steric hindrance. In contrast, in System II the re-circulated percolate was constantly reduced over and over again as it passed through the reducing reactor. Thus oxidation would be diminished and there would be more exposed SH groups available, resulting in higher immobilization and coupling efficiency yields. It is important to note that these yields are similar to those achieved in a batch procedure performed under comparable conditions (applied protein per ml packed gel, room temperature, and pH above 7.0) [5].

After the continuous process performed with System II was finished, the remaining content of SH groups on the solid phase reducing agent was found to be more than 60%. This result suggested great stability of the SH groups on the matrix, since the whole process was carried out at 22 °C and pH 8.5 for 16 h, conditions under which most reductants (e.g. DTT) would be almost completely oxidized.

3.3. Lactolysis

After performing enzyme immobilization using System II, the resulting bio-reactor was fed continuously with skimmed milk at 22 °C at a flow rate of 48 ml/h; steady state lactose hydrolysis (80%) was reached within the first 15 min and remained constant for 24 h. This protocol was carried out four times (between each, the column was washed) and the same steady state percentage of lactose hydrolysis (80%) was maintained throughout.

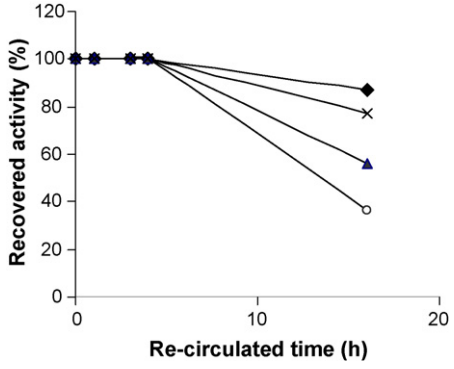


Fig. 4. Stability of reduced enzyme re-circulated under different conditions: (♦) at 4 °C in the absence of gel; (×) at 4 °C during the immobilization process ($EU_{re\text{-}circulated\ percolate} + EU_{washes} + EU_{gel}$); (▲) at 22 °C during the immobilization process ($EU_{re\text{-}circulated\ percolate} + EU_{washes} + EU_{gel}$); (○) at 22 °C in the absence of gel.

4. Conclusions

Since *K. lactis* β-galactosidase requires a previous reduction step before its immobilization onto a thiol-reactive support, the optimization of a continuous solid phase process for reducing and reversibly immobilizing this enzyme onto thiolsulfinate-agarose proved to be an excellent option for developing a fixed-bed bioreactor.

Two Systems were assessed at pH 8.5 (best reduction pH for this yeast lactase) involving mini-reactors connected in series (one containing the solid phase reducing agent and the other packed with the thiol-reactive support) and the resulting immobilization yields and coupling efficiencies were strongly dependent on the reduced enzyme's recirculation process. When this recirculation was performed through both mini-reactors (System II), instead of only through the thiol-reactive mini-reactor (System I), a twofold increase in the immobilization yield and coupling efficiency was achieved.

Compared with batch procedures, this new protocol was characterized by similar reduction degrees and immobilization yields. Moreover, the reduced lactase was directly applied to the thiol-reactive reactor, allowing an easier handling of the enzyme and eliminating intermediate steps (e.g. remotion of the excess of reducing agent before the immobilization process) where the enzyme activity might be affected. In addition, this continuous solid phase process could be more economical, since the entire process saves time, and therefore, costs.

The promising results achieved during lactolysis assays performed at laboratory scale, using an agarose-based bio-reactor obtained with the arrangement according to System II, make this System a very promising approach to develop large scale bio-reactors based on carriers with other mechanical and hydrodynamic properties, suitable for processing skimmed milk and dairy co-products.

Acknowledgments

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References

[1] J.G. Zadow, in: J.G. Zadow (Ed.), Lactose Hydrolysis, Elsevier Applied Science, Essex, England, 1992, pp. 361–408.
[2] R.R. Mahoney, T. Wilder, J. Food Sci. 54 (1989) 899–901.
[3] K. Ovsejevi, B. Brena, F. Batista-Viera, J. Carlsson, Enzyme Microb. Technol. 17 (1995) 151–156.
[4] B. Brena, K. Ovsejevi, B. Luna, F. Batista-Viera, J. Mol. Catal. 84 (1993) 381–390.
[5] K. Ovsejevi, V. Grazú, F. Batista-Viera, Biotechnol. Techn. 12 (1998) 143–148.

[6] F. Batista-Viera, K. Ovsejevi, C. Manta, in: J.M. Guisán (Ed.), Reversible Covalent Immobilization of Enzymes via their Thiol Groups, Humana Press Inc., Totowa, New Jersey, 2006, pp. 185–204.
[7] V. Grazú, K. Cuadra, L. Bentancor, C. Manta, K. Ovsejevi, F. Batista-Viera, Appl. Biochem. Biotechnol. 110 (2003) 23–32.
[8] K. Ovsejevi, V. Grazú, K. Cuadra, F. Batista-Viera, Enzyme Microb. Technol. 37 (2004) 456–462.
[9] R. Axén, H. Drevin, J. Carlsson Acta Chem. Scand. B 29 (1975) 471–474.
[10] K. Brocklehurst, J. Carlsson, M. Kierstan, E. Crook, Biochem. J. 133 (1973) 573–584.
[11] G.L. Ellman, Arch. Biochem. Biophys. 74 (1958) 443.
[12] F. Batista-Viera, C. Manta, J. Carlsson, Biotechnol. Appl. Biochem. 24 (1996) 231–239.
[13] P.K. Smith, R.I. Khron, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klent, Anal. Biochem. 150 (1985) 76–85.
[14] C. Worthington (Ed.), Worthington Enzyme Manual, Freehold, New Jersey, 1993, pp. 179–184.
[15] C. Giacomini, A. Villarino, L. Franco-Fraguas, F. Batista-Viera, J. Mol. Catal. B Enzym. 4 (1998) 313–327.