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Bone-bound enzymes for food industry application

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Abstract

The immobilisation of β-galactosidase and amyloglucosidase (AMG) by means of physical adsorption on to bone powder is described. The influence of the enzyme load, applied to the support on immobilisation, yield and efficiency, has been determined. The immobilisation process improved the thermal stability of β-galactosidase (at 37°C), while immobilised AMG displayed a stability (at 55°C) similar to that of the soluble enzyme. The apparent K_M (K_{app}) values have been determined for both derivatives, for the β-galactosidase derivative K_M was 3 mM using the artificial substrate ONPG and, for the AMG derivative, it was 1 mg/ml using soluble starch as substrate. The extent of lactose hydrolysis achieved, batchwise, with β-galactosidase derivatives acting on lactose buffered solutions, whey, whey permeates and skimmed milk, was in the range of 90% and decreased to 50% after 3 reuses. The conversion of 30% (w/v) liquefied cassava starch achieved with the AMG derivative was 98% until the 11th reuse, and over 95% until the 20th reuse.

Keywords: β-galactosidase; Amyloglucosidase; Enzyme immobilisation; Bone powder; Support

1. Introduction

Many enzymes used in the food industry are quite expensive, but when they are insolubilized by coupling them to an adequate matrix, the resulting biocatalyst may be reused several times, thus lowering the costs (Cheetham, 1985).

Binding of an enzyme to a support may be achieved by chemical or physical means (Kierstan & Coughlan, 1991). Using chemical means, the enzymes are linked to an insoluble matrix by chemical bonds, generally rendering very stable derivatives in which enzyme leakage is prevented. In spite of the high stability of these immobilised systems, covalent attachment is not often used in industrial processes because of the relative complexity of the preparation of such derivatives. Utilisation of non-covalent techniques has been the preferred practice in industrial applications, due to the simplicity and cheapness of this kind of process. Among the non-covalent methods, physical adsorption is the cheapest and the longest established procedure. This fact is of great importance because the industrial usefulness of immobilised biocatalyst depends largely on production of a stable derivative at an economic price.

The nature of the matrix is a very important parameter when evaluating the immobilisation process. Bone is a porous, resistant and relatively cheap material, obtained as a byproduct from meat industries, which meets some of the desired characteristics for a good support. In addition, bone is a natural nontoxic product, which may be used safely in food industries (Findlay, Parkin & Yada, 1986). Several enzymes have been successfully immobilised onto chicken bone particles (Schafhauser & Storey, 1992) and onto pig bone particles (Negishi, Sato, Mukataka & Takahashi, 1989; Mukataka, Negishi, Sato & Takahashi, 1993) through simple adsorption or through post-adsorption enzyme crosslinking. However, so far, bone supports are not commercially available. Because of this, and also because of the impressive growth of the poultry industry both in Ecuador and Uruguay, it seemed reasonable to try to make use of by-products such as chicken necks for the preparation of support material for the immobilisation of enzymes with widespread application in the food industry, such as β-galactosidase (lactase) and amyloglucosidase (AMG). To experiment with this support material, one has to make one's own preparation. β-Galactosidase (EC 3.2.1.23) catalyses the conversion of lactose into glucose and galactose. This enzyme has a broad application to the preparation of

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lactose-hydrolysed products, among them low-lactose dairy products for lactose-intolerant or lactase-deficient people, and to solve pollution problems generated by whey disposal. Amyloglucosidase (EC 3.2.1.3) is an exoenzyme that catalyses the hydrolysis of α-1,4 glycosidic bonds and, to a lesser extent, α-1,6 glycosidic linkages, starting from the non-reducing end of polysaccharides such as starch and similar products, releasing glucose. Glucose syrups of different concentrations can be obtained in this way, and are widely used in food applications where their singular properties, including high fermentability and sweetening power, are very useful. The aim of this work was to assess the use of bone powder as a support material for the immobilisation by physical adsorption of lactase and AMG. Some important characteristics of these enzyme derivatives (such as apparent K_M and thermal stability), as well as the possibility of using them for repeated batch processes, were also assessed taking into account their proposed use in industrial applications.

2. Materials and methods

2.1. Materials

The enzymes β-galactosidase from *Kluyveromices fragilis* (Lactozym 3000 L HP-G), amyloglucosidase from *Aspergillus niger* (AMG 200 L) and thermostable α-amylase (Termamyl 120L) were kindly donated by Novo Industri, Denmark; the PD-10 (Sephadex G-25) columns were purchased from Pharmacia BTG, Uppsala, Sweden; bovine serum albumin (BSA) and the Coomassie Plus reagent were purchased from Pierce, Rockford, IL, USA; the glucose determination kit was purchased from Reacur, Montevideo, Uruguay. Trypsin, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), lactose, glucose and galactose were obtained from Sigma Chemical. Co., St. Louis, MO, USA; skimmed milk, whey and whey permeate were donated by the National Milk Producers Cooperative of Uruguay (CONAPROLE); cassava starch was purchased from the Union of Cassava Producer and Processing Associations of Manabí (UATAPPY), Ecuador. Chicken necks were purchased from a local butchery. All other chemicals used were of reagent grade.

2.2. Methods

2.2.1. Preparation of the supports

2.2.1.1. Bone powder. Primary separation, cleaning and drying — the raw material used in preparing the support consisted of chicken necks, a poultry industry byproduct. Partially clean vertebrae were produced using a manual process, followed by an enzymatic treatment with 0.1% trypsin in 10 mM phosphate buffer pH 8 (at 60°C for approximately 45 min). The material so obtained was treated with a boiling 1% (w/v) sodium hydroxide solution to remove residual proteins from the bone surface and obtain an easily breakable product. This was washed first with a dilute hydrochloric acid solution and then exhaustively with water to neutralise the surface. Finally, it was dried to a moisture content of 4–5% and manually ground in a mortar. Particles ranging in diameter from 50 to 150 μm were selected by sifting.

2.2.1.2. Hydroxyapatite. This was prepared according to the method reported by Siegelman, Wieczorek and Turner (1965).

2.2.1.3. Enzyme activity. β-Galactosidase activity — the activity of the enzyme in solution was tested at room temperature (22°C), using the chromogen ONPG as substrate. A suitably diluted enzyme solution was added to 10 mM ONPG in 50 mM potassium phosphate buffer, pH 7, containing 3 mM $MgCl_2$ (activity buffer I); and the released *o*-nitrophenol (ONP) was determined spectrophotometrically at 405 nm (Worthington, 1988). The immobilised enzyme activity was assayed by incubating 100 μl aliquots of a standard suspension of the derivative (100 mg of suction-dried derivative per ml of suspension), with 2 ml of 28 mM ONPG in activity buffer I, using a 1 cm path cuvette provided with magnetic stirring.

For β-galactosidase, an activity unit (EU) was defined as the amount of enzyme catalysing the hydrolysis of 1 μmol of ONPG per minute under the specified conditions. An extinction coefficient for ONP of 3.5×10^3 M^{-1} cm^{-1} was used.

Amyloglucosidase (AMG) activity — the activity of AMG solution was determined according to the Bernfeld method (1955), at room temperature (22°C) using a 50 mM sodium acetate buffer, pH 4.6 (activity buffer II). The immobilised enzyme activity was assayed, by incubating under end over end rotation, an appropriate amount of suction dried derivative (≈50 mg) with 10 ml of 5% (w/v) soluble starch in activity buffer II. Samples of the reaction mixture (0.4 ml) were withdrawn every 2 min and poured on 0.4 ml dinitrosalicylic (DNS) reagent to stop the reaction. The reducing sugars formed were then quantified (Brena, Ovsejevi, Luna & Batista-Viera, 1993). One amyloglucosidase unit (EU) was defined as the amount of enzyme which releases reducing carbohydrates equivalent to 1 μmol glucose from soluble starch in 3 min at pH 4.6 and at 22°C.

2.2.1.4. Determination of proteins. Protein content in solution was determined by the Coomassie blue G-250 dye-binding method using the Coomassie Plus® reagent, and BSA as a standard (Micro assay procedure,

Pierce Instructions booklet). The immobilised protein was estimated as the difference between the amount of protein applied to the support and the amount of protein recovered in the supernatant and washings. In addition, the protein content of the support was determined by the Kjeldahl method (Tecator Manual, 1979).

2.2.1.5. Immobilisation. The support (1 g) was previously equilibrated in the appropriate activity buffer (I or II) for 1.5 h and incubated under gentle agitation with the enzyme solutions (5 ml) for 1 h at room temperature (22°C). Increasing amounts of β-galactosidase (2.6–11 mg, corresponding to 76 and 322 EU, respectively) and amyloglucosidase (10.7–21.3 mg, corresponding to 801 and 1600 EU, respectively) were applied to the matrix. Excess enzyme was removed by filtration and successive washings with buffer, until no more protein was released. Enzyme activity and protein content in the obtained supernatant and washings were determined.

2.2.1.6. Inactivation kinetics. Inactivation kinetics of free and immobilised β-galactosidase were monitored for 1.5 h at 37°C, using either high load (322 EU/g) or low load (76 EU/g) derivatives. Aliquots (0.2 ml) of each derivative in activity buffer I containing 20 EU or 12 EU were incubated for 30, 60, 120 and 150 min in capped tubes. At the specified times, the relevant tubes were brought to room temperature and the residual activity determined. Similar aliquots of soluble enzyme solution, containing 20 EU, were treated in the same way. For immobilised amyloglucosidase, aliquots of the derivative containing 11 EU were suspended in 1 ml of activity buffer II and the assay was performed at 55°C for 8 h as described above. For soluble enzyme, aliquots contained 8 EU/ml activity buffer II.

2.2.1.7. Determination of K_M *and* K_{app}. K_M and K_{app} values for free and immobilised β-galactosidase were determined using varying concentrations of ONPG (1–12 mM) in activity buffer. For soluble and immobilised amyloglucosidase, the constants were determined using soluble starch as substrate (0.5–2 mg/ml). K_m and K_{app} values were calculated by the Lineweaver-Burk and Eadie-Hofstee methods.

2.2.1.8. Lactose hydrolysis, batch procedure. Aliquots of the β-galactosidase-bone derivative (20 EU) were incubated batchwise at 22°C with 5 and 12% (w/v) lactose solutions in activity buffer I, treated whey (boiled for 10 min, filtered while hot and adjusted to pH 7 with potassium hydroxide), untreated whey, whey permeate and skimmed milk (ratio 1/30 v/v). Samples of the reaction mixtures were withdrawn at different time intervals and filtered to separate the derivative. An aliquot of the filtrate was immediately heated for 5 min in boiling water to stop the reaction. Lactose hydrolysis was monitored by quantifying the amount of glucose formed by an enzymatic method (Trinder, 1969). At the same time, active enzyme release was checked by allowing another aliquot of the filtrate to stand overnight at 4°C and repeating the glucose determination.

Reuse — an aliquot of the derivative (42 EU) after its first use was incubated at 22°C for 4.5 h with 5% (w/v) buffered lactose solution and the amount of glucose produced was determined in the samples taken at different time intervals. The derivative was separated and washed with activity buffer. Then, the washed derivative was used for a second time in the way already described. This protocol was carried out 4 times.

2.2.1.9. Saccharification of cassava starch. The AMG-bone-derivative (6 EU/ml of substrate) was used for producing high glucose syrup at 55°C using 30% w/v cassava starch substrate, previously solubilized with thermostable α-amylase. Follow-up of the reaction was performed for 7 h by quantifying the increase in the reducing power with DNS reagent and by enzymatic glucose determination (Trinder, 1969). At the end of the process the derivative was separated by filtration, washed with activity buffer II and reused 20 times. The ratio between the substrate volume and the suction-dried derivative weight was kept constant for every batch.

3. Results and discussion

3.1. Bone powder as a support for enzyme immobilisation

Fig. 1 shows photographs of the bone support taken before its use in the immobilisation process (Fig. 1a) and after loading it with AMG and its subsequent use in the production of 21 batches of high glucose syrups (Fig. 1b). As shown in the photographs, the bone particles have an irregular shape and their size decreases with usage. Because bone is mainly composed of calcium phosphate and bound protein, the initial particle size may be controlled during support preparation by varying contact time of bones with soda and the reaction temperature. Protein content quantified in several support batches varied between 4 and 8%.

The effect of the addition of increasing loads of β-galactosidase (76–313 EU/g) and amyloglucosidase (800–1600 EU/g) on the immobilisation yield and efficiency of derivatised bone support was studied. For β-galactosidase, the immobilisation yield diminished from 83 to 51% when the enzymatic load was increased (Table 1). At the lowest enzyme load and for those loadings that were well below the support saturation level, all the enzyme activity was preserved; thus 100% of the bound enzyme remained active. From this fact it can be concluded that adsorption of β-galactosidase did not sterically hinder active site groups. However, as

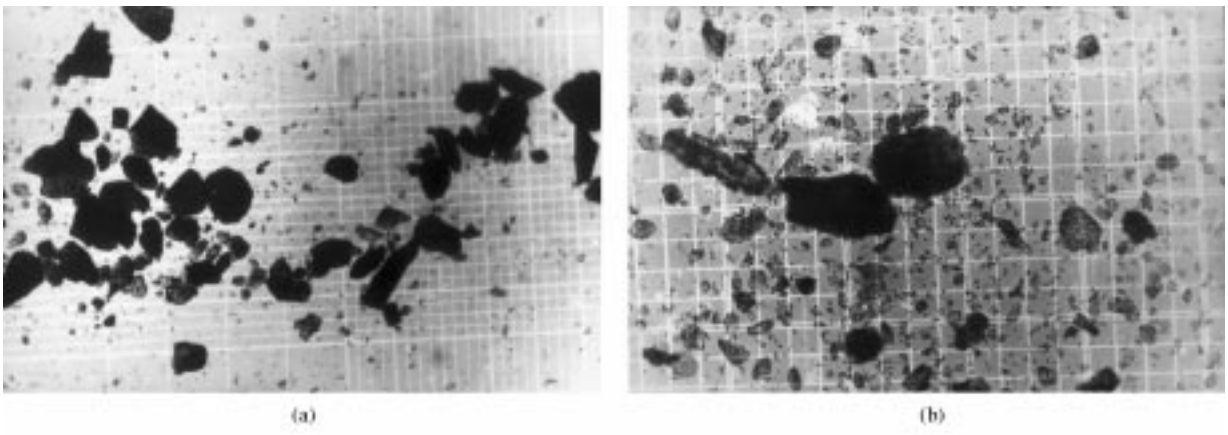

Fig. 1. (a) Support material (bone) prior to use in the immobilisation process. (b) Support after coupling with AMG and after use in production of 21 batches of high content glucose syrup.

Table 1
Influence of the enzyme load on the immobilization of β-galactosidase

Applied activity (EU/g support)[a]	Immobilisation yield (%)[b]	Activity linked to derivative (%)[c]	Immobilisation efficiency (%)[d]
313	51	32	62
267	52	41	78
152	60	57	95
76	83	83	100

[a] Enzyme units (EU) per gram of dry support.
[b] Calculated by taking the difference between applied and recovered activity in the supernatant and washings.
[c] Percentage of total activity applied, measured in the derivative.
[d] Calculated using the expression $E_I/(E_A - E_R) \times 100$; where E_A, is total enzyme activity applied in immobilisation, E_I, is immobilised enzyme activity and E_R enzyme activity remaining in solution.

Table 2
Influence of enzyme load on AMG immobilisation

Applied activity (EU/g support)[a]	Immobilisation yield (%)[b]	Activity linked to derivatives (%)[c]	Immobilisation efficiency (%)[d]
1600	30	6	21
1336	32	8	25
1226	39	8	19
1009	42	9	22
801	51	9	17

[a] Enzyme units (EU) per gram of dry support.
[b] Calculated by taking the difference between applied and recovered activity in the supernatant and washings.
[c] Percentage of total activity applied, measured in the derivative.
[d] Calculated using the expression $E_I/(E_A - E_R) \times 100$, where E_A, is total enzyme activity applied in immobilisation, E_I, is immobilised enzyme activity and E_R enzyme activity remaining in solution.

enzyme loads increased, immobilisation efficiency decreased gradually; this effect can be attributed mainly to diffusion limitations caused by support overload that reduces the access of substrate to active sites (Kierstan & Coughlan, 1991). For AMG adsorption, immobilisation yields diminished from 51 to 30% when the enzyme load applied was increased (Table 2). However, unlike what happened with β-galactosidase, the immobilisation process strongly affected AMG activity for all the loadings studied; thus, expressed activity ranged from 17 to 25% and it was unrelated to the enzyme load applied. These results may be explained either by the fact that immobilisation by adsorption is less specific in the case of AMG, and a real or apparent decrease in activity will ensue if adsorption involves active site groups (Kierstan & Coughlan), and/or by diffusional problems generally produced when immobilised enzymes are acting on macromolecular substrates (starch in this case). The influence of bone particle size on the immobilisation performance of both enzymes was also evaluated.

Experimental data showed that immobilisation yields decreased as particle size of the support increased.

From the above results, it may be concluded that both β-galactosidase and AMG easily bind to bone particles yielding very active derivatives, especially in the case of β-galactosidase. In addition, the simplicity of the immobilisation process (physical adsorption) used in this study, make bone particles a good and economical choice for these applications.

Although bone consists basically of hydroxyapatite crystals embedded in a stable protein matrix (Findlay et al., 1986), neither β-galactosidase nor AMG were adsorbed on to hydroxyapatite gels. This result suggests that enzyme adsorption was mainly due to interactions between the enzyme and the protein components of the matrix (essentially collagen).

3.2. Properties of the enzyme derivatives

The immobilisation process often affects some of the characteristics of soluble enzymes, such as catalytic

activity, thermal stability, kinetic constant (K_M), and optimum pH. These modifications in enzyme properties should be evaluated in order to optimise the immobilised systems for the intended applications.

Thermal stability for free and immobilised β-galactosidase and amyloglucosidase was tested at 37 and 55°C, respectively, temperatures that are consistent with their use on an industrial scale.

The immobilisation process improved the thermal stability of β-galactosidase, especially for the highest-load derivative. Thus, after incubating for 30 min, soluble β-galactosidase lost 65% of its activity while the low-load derivative lost only 30%, and the high-load derivative maintained full activity (Fig. 2). Nevertheless, the stabilisation effect we observed might be the result of loading excess enzyme, which would make the process diffusion-controlled (Adlercreutz, 1993; Brena, 1996).

For amyloglucosidase, there was no improvement in its thermal stability after immobilisation. Thus, remaining activity after incubation for 8 h at 55°C was 40% for immobilised biocatalyst and 55% for free enzyme.

The K_M value for amyloglucosidase was more affected by the immobilisation process than that for β-galactosidase. Thus, the apparent K_M value for the β-galactosidase derivative (3 mM) was only 50% higher than the K_M value for the free enzyme (2 mM), as determined with the artificial substrate (ONPG). In the case of the AMG derivative, its apparent K_M (1 mg/ml) represented a twofold increase compared with the K_M for the soluble enzyme (0.5 mg/ml), using a soluble starch substrate. This value is, however, significantly lower than that (76 mg/ml) reported by Schafhauser and Storey (1992) when immobilising AMG onto granulated chicken bone.

3.3. Applications

Insoluble β-galactosidase and amyloglucosidase derivatives were used batchwise in lactose and starch hydrolysis processes, respectively, at laboratory scale.

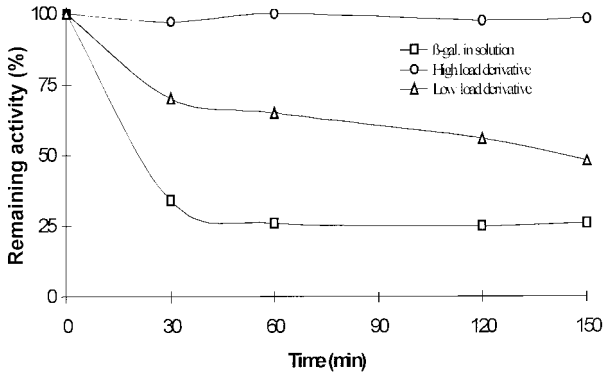

Fig. 2. Thermal stability of β-galactosidase at 37°C.

3.3.1. Lactose hydrolysis

Studies of lactose hydrolysis in buffered lactose solutions (5 and 12%, w/v) were performed batchwise by incubation with β-galactosidase derivative at room temperature (22°C). The degree of conversion attained with the solution of highest concentration (12%) was lower than that attained when using the 5% solution, probably because of the inhibitory effect of the galactose reaction product on the enzyme activity (Fig. 3). A change in temperature from 22 to 37°C produced marked increase in both the rate and degree of lactose hydrolysis (5% solution).

The performance of the β-galactosidase derivative for lactose hydrolysis in treated and untreated whey, whey permeate and skimmed milk was evaluated. The degree of lactose conversion attained at 22°C was similar for all these substrates (Fig. 4), regardless of their composition.

The operational stability of β-galactosidase-bone-derivatives was assessed with buffered lactose solution (5% w/v), batchwise. As shown in Fig. 5, the rate of lactose hydrolysis decreased with the reuse of the derivative, each cycle requiring longer reaction time to reach the same degree of conversion. Thus, at the end of 90 min, the extent of lactose conversion was 85, 60, 30

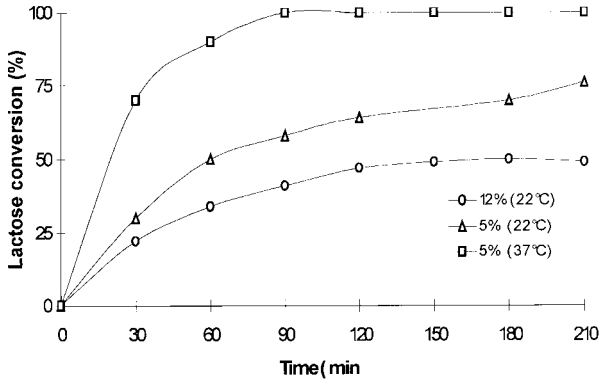

Fig. 3. Hydrolysis of buffered lactose solutions with β-galactosidase-bone derivative.

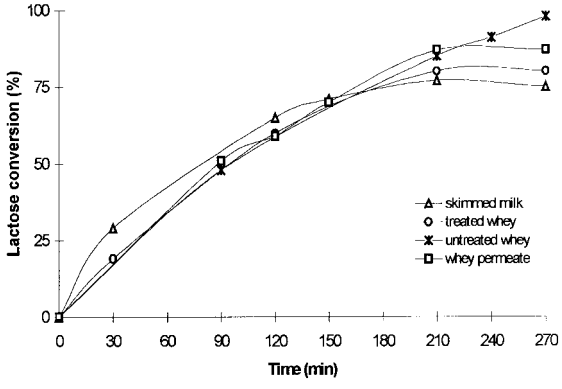

Fig. 4. Lactose hydrolysis of different lactose solutions with β-galactosidase-bone derivative, performed batchwise at 22°C.

and 20% for the first use and successive reuses, respectively. This fall in the activity could not be attributed to the release of the enzyme weakly bound to the support, since the leakage assay performed to determine the release of active enzyme was negative. The loss of activity could be caused by enzyme inactivation, which we were unable to reverse by exhaustive washing with activity buffer I.

3.3.2. Saccharification of cassava starch

The AMG-bone derivative was applied to produce high content glucose syrups, using 30% w/v cassava starch (previously solubilised by thermal-enzyme treatment) as a substrate.

For the highest enzyme load used in the test (6 EU/ml of substrate), the time period required to achieve the maximum conversion (glucose content = 98%) was 3 h (Fig. 6). However, in order to monitor, more easily, the variation in the percentage hydrolysis during 20 reuses of the derivative, the reaction was stopped after 7 h. The ratio between the weight of suction-dried derivative and the substrate volume was kept constant in every batch. The main reason for using an enzyme in immobilised form is that enzyme costs can be diminished, as immobilisation allows the repeated use of the biocatalyst. Therefore, immobilised systems must have good operational stability to be used for long periods of time, in order to improve the economics of the process. Our results demonstrate that the AMG-derivative is very stable, thus, up to the 11th reuse, the glucose content of the resulting syrup was 98%, and for the last batch it was 95% (data not shown). These immobilised enzymes also produce similar glucose concentrations to those produced by soluble enzyme (Reilly, 1985). In our study, AMG-bone derivative showed better operational stability than β-galactosidase derivative.

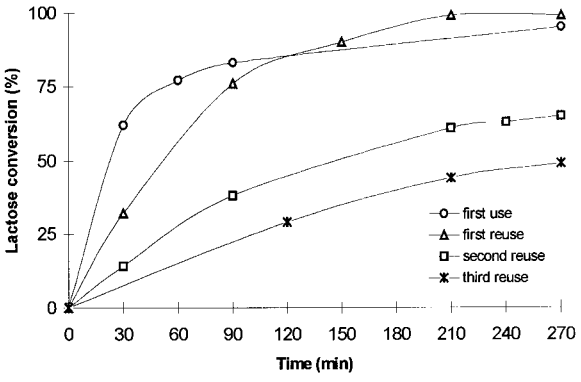

Fig. 5. Reuse of the β-galactosidase-bone derivative for batchwise hydrolysis of 5% (w/v) buffered lactose.

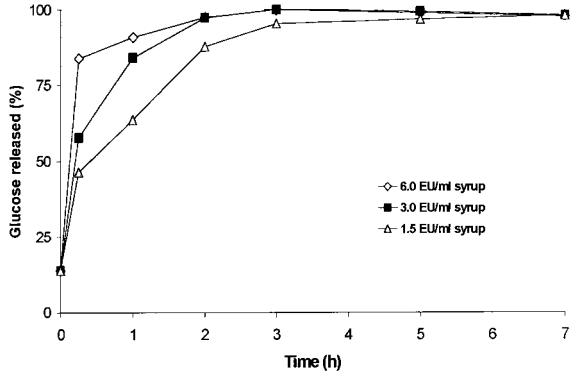

Fig. 6. Saccharification of 30% (w/v) cassava starch with different amounts of AMG-bone derivative.

4. Conclusions

The good immobilisation yields attained during the coupling of β-galactosidase and amyloglucosidase on bone particles, proved that this support meets many of the desired characteristics for immobilisation of enzymes.

The immobilisation process did slightly affect the K_M value for these enzymes; however, the effect was more pronounced for amyloglucosidase for which the K_M value increased twofold. The possibility of using the β-galactosidase-bone derivative in batch and the high bioconversion of lactose attained, make this derivative a useful tool for manufacturing hydrolysed-lactose products. According to our experiments performed on a laboratory scale, the use of AMG-bone derivative allowed a substantial reduction in the reaction times required for producing high-content glucose syrups batchwise (3–7 hours), which for soluble enzyme, under the conditions used in industry, are approximately 48 to 72 h. Furthermore, the high operational stability of the AMG-bone derivative during high-content glucose syrup production was demonstrated throughout twenty reuses, attaining glucose contents in the range of 95–98%.

Acknowledgements

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