ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 4 (1998) 313–327

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Immobilization of β-galactosidase from *Kluyveromyces lactis* on silica and agarose: comparison of different methods

Cecilia Giacomini, Andrea Villarino, Laura Franco-Fraguas, Francisco Batista-Viera *

Cátedra de Bioquímica, Facultad de Química, CC 1157 Montevideo, Uruguay

Received 4 December 1997; accepted 18 February 1998

Abstract

The covalent immobilization of β-galactosidase from *Kluyveromyces lactis* (β-gal) on to two different porous carriers, CPC-silica and agarose, is reported. CPC-silica was silanizated and activated with glutaraldehyde. The activation of agarose via a cyanylating agent (CDAP) was optimized. Gel-bound protein and gel-bound activity were both measured directly, allowing the determination of apparent specific activities (S.A.). Higher amounts of β-gal were immobilized on the activated CPC-silica (maximum capacity, 23 mg ml^{-1} of packed support) than on the CDAP-activated agarose. For the lower enzyme loading assayed (12.6 mg ml^{-1} packed support), 100% of the enzyme was immobilized but only 34% of its activity was expressed. This inactivation during immobilization was confirmed by the S.A. values (22–29 EU mg^{-1} for the CPC-derivatives and 80 EU mg^{-1} for soluble β-gal). The K_{app} (3.4 mM) for the CDAP-derivative with ONPG as substrate was higher than the K_M value for soluble β-gal (2 mM). When the enzyme loading was increased five-fold, the K_{app} increased four-fold, to 13 mM. The V_{app} values for the CPC-derivatives were remarkably lower than the V_{max} for soluble β-galactosidase. CDAP-derivatives showed better thermal stabilities than CPC-derivatives but neither of them enhanced the stability of the soluble enzyme. When stored at 4°C, the activity of both derivatives remained stable for at least 2 months. Both derivatives displayed high percentages of lactose conversion (90%) in packed bed mini-reactors. Glucose production was 3.3-fold higher for the CPC-derivative than for the CDAP-derivative, as a consequence of the higher flow rates achieved.

Keywords: β-Galactosidase; Immobilized biocatalyst; CDAP; Silica; Agarose; Bicinchoninic acid; Lactose hydrolysis

1. Introduction

The dairy industry plays an important role in the economy of farming countries. In Uruguay, an average of 2 million liters of milk are processed per day, of which approximately one fifth are for cheese manufacturing, generating around 360 m^3 of whey day^{-1}. Part of this whey undergoes ultrafiltration to recover proteins, thus, generating a whey permeate containing mainly lactose (4.5% w/v), minerals and vitamins. However, large quantities of whey are still discarded, creating environmental pollution and also wasting an important potential source of nutrients.

β-Galactosidase (E.C. 3.2.1.23), also called lactase, catalyzes the hydrolysis of β-galactosidic linkages such as those present in lactose to

* Corresponding author. Fax: +598-2-9241906; e-mail: bioquim@bilbo.edu.uy.

1381-1177/98/$19.00
PII S1381-1177(98)00071-X

yield glucose and galactose. The use of β-galactosidase has been suggested for hydrolysis of the lactose in dairy products and in whey byproducts, so that the resulting sugars may be used for growing baker's yeast [1]. Lactose conversion would, in addition, help to solve the pollution problem caused by whey disposal.

Although soluble β-galactosidase can be used for lactose hydrolysis, immobilization of the enzyme on a solid support offers additional advantages. Economic considerations indicate that the use of immobilized β-galactosidase for lactose hydrolysis in whey is economically feasible in spite of the cost of the enzyme and of the immobilization process [2]. This is mainly due to the fact that the enzyme derivative can be reused several times, and to the possibility of developing a continuous hydrolysis process, which releases free enzyme product. A large number of different supports have been used for immobilization of lactases [3,4].

Most commonly, protein immobilization techniques through covalent coupling involve the reaction of protein amino groups with electrophilic moieties introduced on a solid support. For inorganic supports, such as silica, derivatization can be performed by silanization with trialkoxysilanes containing an organic functional group, such as an amino group [5]. One versatile silane compound is 3-aminopropyltriethoxysilane; the obtained solid phase—alkylamine can then be activated with glutaraldehyde to yield an active carbonyl derivative, by the formation of a Schiff's base.

CNBr-activated agarose has been extensively employed for the immobilization of sensitive ligands such as proteins using mild conditions, even though the yield of immobilized protein is very low. However, its use involves serious health hazards. Kohn and Wilcheck [6] have devised a method which increases reactivity, and thus yield, by formation of cyanotransfer complexes between CNBr and certain bases. One of the most frequently used cyanotransfer complexes is 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP). The main advantages of this reagent are its safety during handling, stability and easy storage at room temperature for long periods.

In general, in the majority of immobilization procedures involving formation of covalent bonds, catalytic capability of the enzyme is often noticeably affected. The immobilization often cause a dramatic change in the apparent parameters of the enzyme-catalyzed reaction and in the thermal stability. The extent of these changes depends not only on the immobilization method, but also on the reaction system used. Therefore, any new immobilized enzyme derivative must be carefully characterized with respect to all these parameters, and its overall performance evaluated with regard to the final purpose for which the biocatalyst was intended.

The aim of our study was to contribute, by the development of new enzyme derivatives, to the solution of the technological problems referred above. To this end, β-galactosidase from *Kluyveromyces lactis* was immobilized on to two different carriers: an organic one, agarose activated with CDAP, and an inorganic carrier, CPC-silica silanized and activated with glutaraldehyde. The activation and immobilization procedures were optimized and the derivatives obtained were characterized with regard to the effects on enzyme activity of the coupling methods and enzyme loads used. Kinetic parameters, thermal stability and lactose hydrolysis were evaluated as they are all of considerable value for design and development of industrial reactors using immobilized enzyme for lactose conversion.

2. Materials and methods

Lactose monohydrate, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), 2,4,6-trinitrobenzene sulfonic acid (TNBS) and β-mercaptoethanol (β-MSH) were purchased from Sigma (St. Louis, MO, USA). Maxilact LX 5000, a liquid preparation of yeast lactase derived from *K. lactis*, was kindly supplied by Gist-Brocades

Food Ingredients (Seclin, Cedex, France). 1-Cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP) and Sepharose 4B were supplied by Pharmacia Biotechnology (Uppsala, Sweden). A glucose determination kit was purchased from Reacur (Reactivos del Uruguay, Uruguay). Triethylamine (TEA), CPC-silica carrier (30–45 mesh; pore size 375 Å), 50% glutaraldehyde and 3-aminopropyltriethoxysilane were purchased from Fluka (Buchs, Switzerland). BCA protein assay reagents were from Pierce (Rockford, IL, USA). Whey permeate was kindly supplied by CO. NA. PRO. LE. (Cooperativa Nacional de Productores de Leche, Uruguay). All other chemicals used were reagent or analytical grades.

2.1. Matrix activation

2.1.1. Activation of CPC-silica

CPC-silica carrier (10 g) was first cleaned by boiling it in 5% nitric acid solution for 45 min, followed by exhaustive washing with distilled water to remove any residual acid. Then it was washed with acetone on a glass filter funnel and dried at 60°C for 30 min. The clean, dry inorganic support material was added to 45 ml of 3-aminopropyltriethoxysilane (10% w/v) dissolved in acetone. The solution was evaporated to dryness leaving behind the silane adsorbed to and reacted with the support material, and was then washed with acetone to remove unreacted silane reagent. The product was dried at 110°C for 4 h [5]. Free amino groups bonded to the solid support were quantified by titration with TNBS, according to the method of Antoni et al. [7]. This inorganic support containing 86 μmol of primary amino groups per ml of packed gel, previously equilibrated with 20 mM potassium phosphate buffer pH 7.0, was then activated with 140 ml of 2.3% glutaraldehyde solution. The reaction was allowed to continue for 1 h with slow rotation at room temperature, followed by extensive washing with the same buffer, and it was finally equilibrated with 0.1 M potassium phosphate buffer pH 7.0 containing 2 mM magnesium chloride and 0.1 M potassium chloride (immobilization buffer). The activated CPC-carrier was immediately used for immobilization.

2.1.2. Activation of Sepharose

Sepharose 4B was activated essentially according to Kohn and Wilcheck [6] and Carlsson et al. [8] with the following modifications: 3 g of swollen Sepharose 4B was washed on a glass filter with 50 ml of water, 50 ml of acetone:water (3:7 v/v) and then with 50 ml of acetone:water (6:4 v/v), previously cooled to 4°C. The gel was drained by mild suction and then it was transferred to a 10-ml glass beaker and mixed with 3 ml of acetone:water (6:4 v/v) pre-cooled to 4°C. An amount of 75 mg of CDAP dissolved in 2 ml of acetone:water (6:4 v/v) was added to the gel suspension under vigorous stirring at 4°C for 3 min; 360 μl of 0.2 M TEA solution was added dropwise in a period of 1 to 2 min. After 3 min, the entire reaction mixture was quickly added to 50 ml of ice-cold 50 mM HCl. After no more than 2 min the gel was rapidly transferred to a glass filter funnel and washed with 50 ml of ice-cold water. The activated gel (CDAP-agarose) was equilibrated with 20 mM potassium phosphate buffer pH 7.0 containing 2 mM magnesium chloride and 0.1 M potassium chloride (activity buffer), and immediately used for coupling to β-galactosidase.

2.2. Immobilization of β-galactosidase from K. lactis (β-gal)

Aliquots of the activated carriers prepared as described above were incubated with solutions containing different concentrations of β-gal diluted in the immobilization buffer for the activated CPC-silica, and diluted in the activity buffer for the CDAP-activated agarose. The ratio between the volume of the enzyme solution and the suction dried gel weight was kept constant (7.2 and 6.3 ml g^{-1} of suction dried gel for the activated CPC-silica and CDAP-activated agarose, respectively). The suspensions were

gently agitated at room temperature for 20 h for the activated CPC-silica and for 4 h for the CDAP-activated agarose. The immobilized β-gal on the activated carriers, termed CPC-derivative and CDAP-derivative, respectively, were exhaustively washed with the buffer used for the immobilization step, then equilibrated with the activity buffer and stored at 4°C until use.

2.3. Protein determination

The protein content of the CPC-derivatives was determined by total amino acid analysis after extensive drying over P_2O_5 in a dessicator until they reached a constant weight, followed by hydrolysis in 6 N HCl for 24 h at 110°C. This method is very sensitive and precise but it requires special instrumentation. Another option is the bicinchoninic acid (BCA) assay developed by Smith et al. [9]. The method combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous ion using the bicinchoninic acid. The interaction of two molecules of BCA with one Cu^{+} gives a purple product which is water soluble and absorbs strongly at 562 nm. The BCA method has been used with soluble protein samples [9], with proteins adsorbed to microtiter plates [10,11] and in the case of protein covalently bound to agarose [12], among others. The protein content of the CDAP-derivatives was determined in all cases by total amino acid analysis and by the BCA method. The concentration of soluble β-galactosidase was determined by the BCA method. A calibration curve using BSA as standard was established by incubating 100 μl aliquots of appropriate dilutions (concentrations between 0.02–0.2 mg ml^{-1}) with 2 ml of BCA working reagent for 15 min at 60°C. For the immobilized protein, the following protocol was applied: suction dried gel derivatives were washed with distilled water on a glass filter. An amount of 100 mg of suction dried gel was suspended in 1.0 ml of distilled water (suspension I). An aliquot of 100 μl of this suspension I (10 mg of suction dried gel) was transferred, under continuous stirring and using an automatic pipette in which the plastic tip had been cut to enlarge the inlet opening, to 1.0 ml of distilled water (suspension II). An aliquot of 100 μl of suspension II (1 mg of suction dried gel) was taken during stirring and incubated with 2 ml of BCA reagent for 15 min at 60°C in a water bath provided with a shaker. The gel suspensions were cooled to room temperature and the absorbance of the supernatants were measured at 562 nm. For those samples with a low content of immobilized enzyme, 100 μl of suspension I (10 mg of suction dried gel) was incubated directly with 2 ml of BCA reagent. Sepharose 4B was treated exactly as each of the samples containing immobilized protein, and used as a blank. The absorbance of blanks and each of the derivative samples was determined at least in triplicate. In parallel, aliquots of 0.5 g of suction dried gels were put in a dessicator over P_2O_5 for 1 week, and subsequently used in some cases for amino acid analysis.

We determined that 1.00 g of filter dried Sepharose 4B corresponds to 1.55 ml packed gel and 0.05 g of dry gel. In the same way, 1.00 g of filter dried CPC-silica carrier corresponds to 1.03 ml packed support and 0.56 g of dry support.

For both, the CPC- and CDAP-derivatives, the gel-bound protein was expressed as (i) mg of protein ml^{-1} of packed gel, and (ii) amount of immobilized protein as a percentage of the amount of protein initially applied.

2.4. Enzyme activity

The activity of β-galactosidase was assayed using the chromogen ONPG as substrate. A suitably diluted enzyme solution was added to a solution of ONPG in activity buffer (14 mM) at 25°C, and the rate of formation of free *o*-nitrophenol (ONP) was recorded spectrophotometrically using a 1-cm path length cuvette

provided with magnetic stirring [13]. One unit of enzyme activity (EU) was defined as the amount of enzyme hydrolyzing 1 μmol of substrate min^{-1} in the conditions defined above. An extinction coefficient for ONP of 3.5×10^3 M^{-1} cm^{-1} was used [14]. For the immobilized enzyme, activity was measured under identical conditions by incubating appropriate aliquots of the gel suspensions with the substrate solution mentioned above.

Enzymatic activity was expressed as EU ml^{-1} for the soluble enzyme. The gel-bound activity was measured directly in the derivative and it was expressed as (i) EU ml^{-1} of packed gel and (ii) amount of immobilized activity as a percentage of the amount of activity initially applied.

The specific activity (S.A.) was defined: (i) as the ratio between the activity expressed in EU ml^{-1} and the concentration in mg of protein ml^{-1}, for the soluble enzyme; (ii) as the ratio between the activity expressed in EU ml^{-1} of packed gel and the concentration of immobilized protein in mg ml^{-1} of packed gel, for the immobilized enzyme.

2.5. Determination of kinetic parameters (K_M and V_{max})

Kinetic parameters were determined using varying concentrations of ONPG (0.5–28.0 mM) in activity buffer. The K_M and the V_{max} were determined by Lineweaver–Burk, Eadie–Hofstee and direct linear plot methods [15].

2.6. Temperature stability

Aliquots (0.5 ml) of gel suspensions containing 0.28 mg ml^{-1} of gel suspension for the CDAP-derivative and 0.35 mg ml^{-1} of gel suspension for the CPC-derivative were incubated for 30 min at 21°C, 40°C, and 50°C. They were brought to room temperature and the residual activity was determined. Soluble enzyme solution containing equivalent amounts of protein were treated in the same way.

2.7. Temperature stability at 45°C

Immobilized enzyme suspensions containing 3.27 mg protein ml^{-1} of standard suspension for the CPC-derivatives, 0.35 mg protein ml^{-1} of standard suspension for the CDAP-derivative, and enzyme solutions containing equivalent protein concentrations were incubated in activity buffer at 45°C under continuous stirring. Aliquots were taken at regular intervals, brought to room temperature, and the residual activity was determined.

2.8. Lactose hydrolysis

The enzyme derivatives were evaluated for their ability to hydrolyze lactose, and the glucose produced was measured by an enzymatic method.

The percentage of lactose conversion was defined as the ratio between the concentration in mg ml^{-1} of glucose produced and the concentration in mg ml^{-1} of glucose theoretically produced by 100% hydrolysis of the substrate.

2.8.1. Whey permeate treatment

The whey permeate was prepared for use as a substrate by boiling it for 10 min, filtering, cooling to room temperature and adjusting to pH 7.0 with KOH.

2.8.2. Lactose hydrolysis performed batchwise

Aliquots of 0.5 ml of enzyme solution (containing 8 EU ml^{-1}), CPC-derivative and CDAP-derivative (containing 8 EU ml^{-1} of standard suspension), were added to (i) 5 ml of 4.75% of lactose solution dissolved in the activity buffer and (ii) 5 ml of whey permeate. At given time intervals up to 4 h, aliquots of the reaction mixture were taken and heated for 5 min in boiling water to stop the reaction. Lactose hydrolysis was monitored by measuring the amount of glucose released, by an enzymatic method.

2.8.3. Lactose hydrolysis performed in a mini-reactor system

In our continuous hydrolysis system, columns of 16 mm of diameter were prepared by packing with 8.10 ml of packed CDAP-derivative (containing 859 total EU) or 5.15 ml of packed CPC-derivative (containing 5527 total EU). Lactose, 4.75% (w/v) in activity buffer was pumped through the columns for 30 h at flow rates of 30 ml h^{-1} and 110 ml h^{-1}, respectively. Whey permeate was also pumped into a CDAP-derivative column in the same experimental conditions. Aliquots of 1 ml were taken directly from the output of the columns at regular time intervals, boiled for 5 min in water bath, cooled, appropriately diluted and assayed for glucose content.

The columns were unpacked, the gel derivatives were washed with the activity buffer and stored at 4°C; later, they were repacked and reused for lactolysis for a further 25 h with the CPC-derivative, or for a further 50 h with the CDAP-derivative.

3. Results

3.1. Determination of solid phase-bound protein

Total amino acid analysis and BCA methods were used in this work, for direct quantification of the solid phase-bound protein. For reliable results, the solid matrices without functional groups or ligands should not themselves contribute to color formation. The agarose and the CPC-silica matrices we used proved not to develop color with the BCA reagent (absorption values < 0.02). The assay method requires a single incubation of the solid support with the BCA assay reagent at 60°C. Color formation occurs in solution, and the supernatant is later easily separated from the solid. In validating our BCA protocols, pure Concanavalin A was used as the model protein, and it was immobilized on to Sepharose by the CDAP-activation procedure. Several derivatives with different gel-bound protein contents were prepared and each one was analyzed by both total amino acid analysis and by the BCA method (Table 1). The correlation coefficient between these two methods was 0.994. We have adapted the BCA method to measure the covalent immobilization of a commercial enzyme (β-galactosidase from *K. lactis*) on to agarose and silica supports. On one of the CDAP-derivatives, the immobilized β-galactosidase content was found to be 1.06 mg ml^{-1} packed gel by total amino acid analysis and 0.97 mg ml^{-1} packed gel by BCA method.

For protein determination by the BCA method in the case of the CPC-derivative, it was necessary to reduce the remaining aldehyde groups on to the matrix with sodium borohydride [16]. The reduction procedure was carried out at 10°C for 30 min, using 1 mg of sodium borohydride ml^{-1}. During this reduction, protein leakage was detected. When determined by the BCA method, the gel-bound protein content after reduction was 24.3 mg ml^{-1} packed gel. The initial protein content was 36.4 mg ml^{-1} of packed gel, as determined by total amino acid analysis. The CPC-silica derivative was also reduced under more drastic conditions: at room temperature, incubating for 24 h and using 2 mg ml^{-1} of sodium borohydride. In these conditions, the protein content of the derivative was 16.0 mg ml^{-1} packed gel. The protein leakage which was observed during reduction of the CPC-derivative, could be due to instability of the silica carrier at the alkaline pH used for the

Table 1
Comparison of quantities of gel-bound protein determined by total amino acid analysis and by the BCA method, for Con A–Sepharose derivatives

Total amino acid analysis (mg g^{-1} dry gel)	BCA method (mg g^{-1} dry gel)
16.4	17.2
78.5	86.7
128.2	133.6
230.0	218.0
259.3	269.0
272.7	260.0

reduction process and/or instability of the enzyme at high pH. For these reasons, the reduction step was omitted during the immobilization of β-gal on to activated CPC-silica.

3.2. Immobilization of β-galactosidase and effect of protein load

The matrices used, CPC-silica and Sepharose 4B, are macroporous particulate supports with pore diameters of 375 Å and 300 Å, respectively [17]. These porous carriers have a high surface area per unit weight allowing high protein capacity. The methods used to activate the matrices (activation of agarose by CDAP and silanization of CPC-silica followed by treatment with glutaraldehyde) are straightforward and easy to perform. During the activation of agarose with the CDAP reagent [6,8], the technique was adjusted to maximize the amount of β-galactosidase immobilized on the matrix. The incubation time between the agarose and the CDAP was lengthened from 30 s to 2 min, and the washing step with 50 mM HCl was adjusted in order to obtain more reactive cyanate groups on the agarose. This washing step is required to hydrolyze from the gel the pyridinium isourea derivative, formed as a byproduct of the activation reaction. Although the active cyanate groups should not be affected by this treatment, we observed that when the gel was washed with 50 mM HCl for 2 min, the resulting activated agarose was able to immobilize six-fold more protein than when it had been washed for 15 min as originally described by Kohn and Wilcheck [6].

A control with the soluble enzyme was assayed in parallel with enzyme immobilization on both CDAP- and CPC-silica activated matrices in exactly the same conditions of temperature, pH, incubation period, and ionic strength. This control allowed us to determine whether the cause of any enzyme inactivation occurring during the immobilization was due to the reaction conditions, or to other factors inherent to the immobilization procedure (for example, due to a special microenvironment generated within the matrices or structure modification arising from the covalent attachment of the enzyme to the carrier). The soluble enzyme control in these conditions maintained 100% of its initial activity, thus, confirming that the immobilization methods we selected work in mild conditions which are compatible with optimal enzyme performance.

To observe the effect of protein content on the activated supports, the enzyme load was varied over a wide range. For both CPC- and CDAP-derivatives, similar enzyme loads, of between 20 and 200 mg of protein g^{-1} of dry support, were assayed. For operational and practical purposes, it is more convenient to express the solid-phase bound protein and the solid-phase bound enzyme activity per ml of packed gel; values are so expressed in Tables 2 and 3. Table 2 shows that the capacity of the CPC-silica carrier for the β-gal is about 23 mg ml^{-1} packed gel. Besides, for the CDAP-activated

Table 2
Effect of enzyme load on the β-gal-CPC-derivative

Applied protein (mg ml^{-1} packed gel)	Gel-bound protein (mg ml^{-1} packed gel)	(%)[a]	Gel-bound activity (EU ml^{-1} packed gel)	(%)[b]	Gel-bound S.A. (EU mg^{-1} protein)
12.6	12.6	100	333	34	26
23.2	18.6	80	537	29	29
46.4	21.9	47	644	18	29
92.8	23.0	25	513	8	22

Specific activity (S.A.) of the soluble enzyme was 80 EU mg^{-1} protein.
[a]Amount of immobilized protein as percentage of the amount of protein initially applied.
[b]Amount of immobilized activity as a percentage of the amount of activity initially applied.

Table 3
Effect of enzyme load on the β-gal-CDAP-derivative

Applied protein (mg ml^{-1} packed gel)	Gel-bound protein (mg ml^{-1} packed gel)	(%)[a]	Gel-bound activity (EU ml^{-1} packed gel)	(%)[b]	Gel-bound S.A. (EU mg^{-1} protein)
1.0	1.0	100	82	112	82
1.5	1.4	94	143	113	102
2.8	2.5	88	219	107	88
6.9	5.4	78	378	77	70

Specific activity (S.A.) of the soluble enzyme was 80 EU mg^{-1} protein.
[a]Amount of immobilized protein as a percentage of the amount of protein initially applied.
[b]Amount of immobilized activity as a percentage of the amount of activity initially applied.

agarose (Table 3), even at the highest enzyme load assayed, the full capacity of the support was not occupied, and free reactive groups remained. For all the protein loads assayed, each CPC-derivative yielded higher percentages for gel-bound protein than for gel-bound activity. At the lowest enzyme load, the immobilized protein determined was 100%, whereas the expressed activity for this derivative was 34%. Thus, the lower specific activity of this derivative (26 EU mg^{-1} protein) when compared with the specific activity value for the soluble enzyme (80 EU mg^{-1} protein), is evidently due to partial enzyme inactivation occurring during the immobilization process. This inactivation effect is not observed in the case of CDAP-derivatives for which specific activity values are closely similar to those obtained for the soluble enzyme control.

3.3. Determination of kinetic parameters (K_M and V_{max})

Kinetic parameters for β-galactosidase derivatives were determined using ONPG as substrate and the data were processed using different methods: direct linear plot, Lineweaver–Burk and Eddie–Hofstee plots. The values obtained by direct linear plot are summarized in Table 4. The apparent K_M (K_{app}) values for the CPC-derivative were three-fold higher than the K_M value (2 mM) for the soluble enzyme and it remained unchanged for the different assayed enzyme loadings. For the CDAP-derivative with lowest protein content, the K_{app} is twice as great as that of the K_M value of the soluble enzyme. When analyzing the K_{app} values obtained for the different CDAP-derivatives, we observed that by increasing the enzyme load on the derivative five-fold, the K_{app} increased four-fold and, in comparison with the K_M value for the soluble enzyme, it was proportionately increased (by 6.5-fold). It must be borne in mind that the amount of the enzyme immobilized on the CDAP-derivative was consistently lower than the enzyme loading on the CPC-derivative, in our hands. The apparent V_{max} (V_{app}) values determined for the CPC-derivatives were markedly lower than the V_{max} of the soluble enzyme (91 μmol_{ONP} min^{-1}

Table 4
Kinetic parameters for the CPC- and CDAP-derivatives of β-galactosidase, determined using ONPG as substrate

CDAP-derivative			CPC-derivative		
Gel-bound protein (mg ml^{-1} packed gel)	K_{app} (mM)	V_{app} (μmol ONP mg^{-1} min^{-1})	Gel-bound protein (mg ml^{-1} packed gel)	K_{app} (mM)	V_{app} (μmol ONP mg^{-1} min^{-1})
1.0	3.4	104	12.6	6.3	31
1.4	5.8	150	18.6	5.8	36
2.5	8.1	133	21.9	6.3	42
5.4	13.0	142	23.0	7.5	40

mg^{-1} protein). This result is consistent with the respective specific activities of these derivatives compared to the soluble enzyme, which indicates that inactivation of the enzyme was occurring during the immobilization procedure, as discussed above. For the CDAP-derivatives, the determined V_{app} were higher than the V_{max} of soluble enzyme (Table 4).

3.4. Thermal stability

The thermal stability of the CPC- and CDAP-derivatives was investigated at various temperatures, analyzed and compared with the thermal stability of the soluble enzyme (Table 5). The CPC-derivative was less stable than the soluble enzyme even at 40°C while the CDAP-derivative displayed the same stability as that of the soluble enzyme. When the thermal inactivation kinetics were studied at 45°C, the CPC-derivative was less stable than the free enzyme (Fig. 1). The same experiment was also performed with a β-gal-CPC derivative treated with sodium borohydride. Treatment with sodium borohydride blocks the remaining active groups by reducing them to alcohols, and also reduces the Schiff's base linking the enzyme and the activated support. Within the first 10 min of incubation, we observed an increased stability of the borohydride treated-derivative with respect to the unblocked derivative. After this period, the kinetic profile at 45°C of the borohydride treated-derivative showed a degree of inactivation similar to that exhibited by the unblocked derivative (data not shown). The CDAP-derivative exhibited almost the same thermal inactivation profile as that of the free enzyme (Fig. 2). When stored at 4°C the bound activity of both CDAP- and CPC-derivatives remained intact for at least 2 months.

Table 5
Thermal stability of free and immobilized β-galactosidase

Temperature (°C)	Remaining activity (%)[a]		
	Free enzyme	CPC-derivative	CDAP-derivative
21	98	100	100
40	95	60	107
50	26	7	34

[a]After 30 min of incubation.

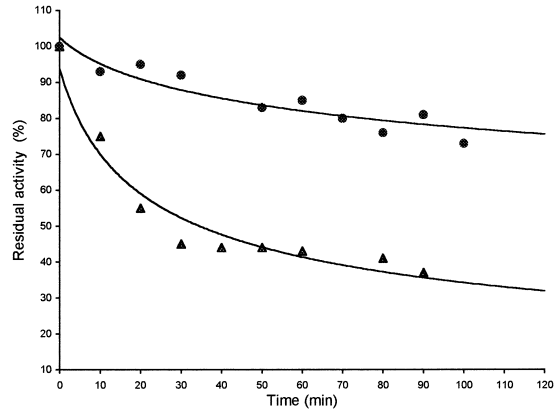

Fig. 1. Inactivation kinetics at 45°C. Free enzyme (3.9 mg protein ml^{-1}) (●); CPC-derivative (3.3 mg protein ml^{-1} of suspension) (▲).

3.5. Lactose hydrolysis

The performance of the β-galactosidase derivatives for lactose hydrolysis of buffered lactose solutions (4.75% w/v) and of whey

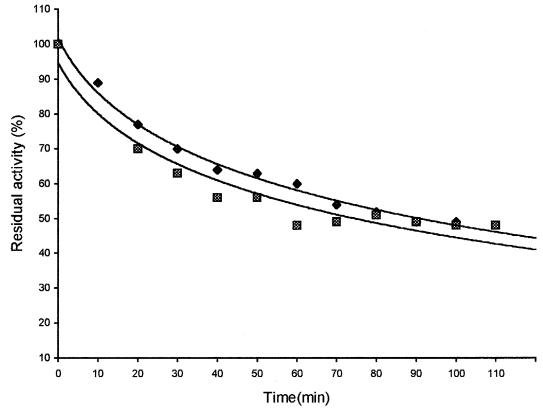

Fig. 2. Inactivation kinetics at 45°C. Free enzyme (0.38 mg protein ml^{-1}) (◆); CDAP-derivative (0.35 mg protein ml^{-1} of suspension) (■).

permeates, was evaluated. In batch experiments, the CPC-derivative was incubated with buffered lactose solution and with whey permeate. The percentage of lactose conversion (lactolysis) achieved in both these cases was 90% after 2 h. The same degree of conversion was obtained in a parallel lactolysis experiment, carried out with an equal amount of soluble β-gal. When the CDAP-derivative was incubated batchwise with lactose solution, lactose conversion reached 95% after 4 h, while 75% lactose conversion was achieved in the same period, when whey permeate was used as the substrate. The performance of the CDAP-derivative was identical to that of the soluble enzyme, as assayed in parallel experiments.

Lactolysis was also carried out in packed bed mini-reactors. Table 6 shows that very high percentages of lactose hydrolysis were quickly reached when using columns packed with either CDAP- or CPC-derivatives. These rates of lactose conversion remained constant for the entire test period of 25 h. Volumes of 2775 ml, and 750 ml, of lactose solution were pumped into the columns containing CPC- and CDAP-derivatives, respectively. No leakage of active enzyme was detected during these processes. After their first use, the columns were thoroughly washed with activity buffer, and the remaining gel-bound activity was determined. Over 75% of the initial EU, determined using ONPG as substrate (EU_{ONPG}) still remained in the gel derivatives (Table 6). After their first use, the derivatives were re-packed, equilibrated with activity buffer and fresh lactose solutions were again pumped through the columns. Once again, high percentages of lactose conversion were quickly reached when operating for 25 h in the case of CPC-derivative, and for 50 h in the case of the CDAP-derivative. After this second use of the mini-reactors, no further enzyme inactivation was observed in the case of the CDAP-derivative, but the CPC-derivative lost 67% of the enzyme activity that was present at the beginning of the second lactolysis cycle.

The ability of the CDAP-derivative to hydrolyze lactose was also analyzed using whey permeate as a substrate. In this experiment lactose conversion of 90% was soon reached, and remained constant for 30 h (Fig. 3). At the end of the experiment, 85% of the initial immobilized enzyme activity still remained in the gel. The column was reused by pumping fresh whey permeate through it and the reactor was operated continuously for 53 h. When analyzing its performance with respect to time, 86% lactose conversion was observed for the first 27 h of continuous performance. This percentage decreased to 47% for the final 26 h. Throughout the whole second use, a decrease of only 6% in the EU_{ONPG} of the β-gal was detected. Presumably, the whey permeate exerts an undesirable

Table 6
Performance of immobilized β-galactosidase in lactose hydrolysis

Column packed with	Lactose conversion[a] (%)	Pumped volume (number of bed volumes)	Total activity (EU_{ONPG}) after column use	
CPC-derivative			*EU*	%
1st use (25 h)	90	540	4145	75
Reuse (25 h)	90	540	1382	25
CDAP-derivative				
1st use (25 h)	100	93	670	78
Reuse (50 h)	90	214	670	75

Lactolysis was performed using 4.75% lactose in activity buffer (see Section 2).
[a]Maximal percentage of lactose conversion with respect to theoretical total conversion (= 100%) that was achieved in 1 h and remained constant throughout the process.

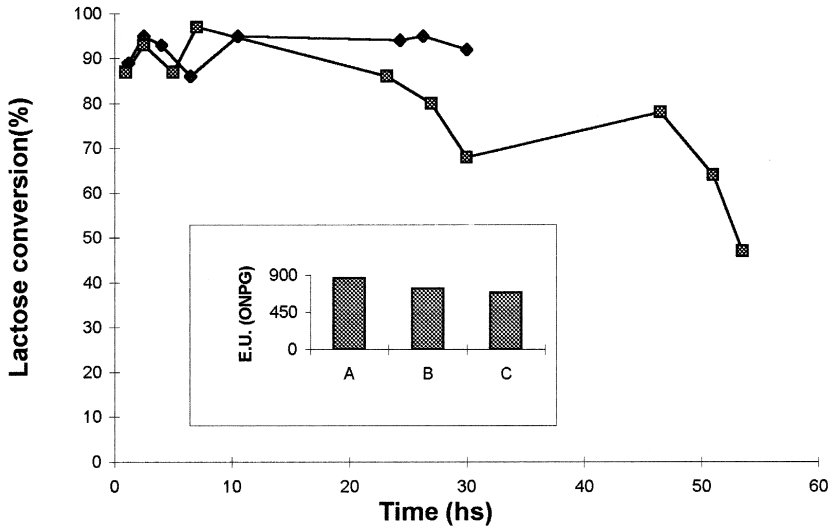

Fig. 3. Performance of the CDAP-derivative in continuous lactolysis process carried out with whey permeate as substrate. First use (♦); reuse (■); (A) initial activity (EU_{ONPG}); (B) activity (EU_{ONPG}) after the first use; (C) activity (EU_{ONPG}) after reuse.

effect on the CDAP-derivative which shortens the useful lifetime of the reactor. The nature of this putative effect is not known.

4. Discussion

Two immobilized biocatalysts were prepared by covalent attachment of β-galactosidase from *K. lactis* on to two different carriers. Agarose and CPC-silica, the matrices we used, are representative of two major classes of supports. CPC-silica belongs to the inorganic carriers, which have been used in some of the earliest research work on protein immobilization. Agarose belongs to the group of organic matrices [18]. Due to their high reactivity, organic supports have been used more widely in the development of immobilized enzymes [19]. Inorganic carriers, due to their physical properties, are suitable for industrial use, and they offer some advantages over their organic counterparts, such as their ability to withstand high mechanical strength, thermal stability, ease of handling, long shelf life, high flow rates in continuous reactors, and the ease with which they can be regenerated by simple pyrolysis, among others. Their structure remains unaltered over wide ranges of pressure and temperature, and they also have a relatively wide useful pH range. Most of the surface available for protein coupling to these porous supports is the internal surface; they have an internal morphology that not only allows the protein to be covalently linked to the matrix, but also offers easy access to soluble molecules, thus, minimizing diffusional effects. Moreover, proteins bound on the internal surface may be protected from turbulence and the harsh external environment [19].

In spite of the fact that agarose and CPC-silica are both porous matrices, higher amounts of β-galactosidase were immobilized on activated CPC-silica than on CDAP-activated agarose. We could not saturate the CDAP-derivative with the enzyme loads we used, although our results with the CPC-derivative indicated that near maximal capacity for the enzyme immobilization was achieved. The higher capacity of the CPC-silica compared with the agarose may be due to the fact that the silica carrier expose a greater surface area (32 m^2 ml^{-1} support) than does Sepharose (5 m^2 ml^{-1} support), thus, pro-

viding a greater number of immobilization sites for the enzyme [17]. The number of reactive groups introduced into the matrix may also be responsible for the higher capacity of the CPC-derivative.

There are only a few methods described in the literature for the determination of total support-bound protein. Frequently, protein immobilization yields are estimated indirectly, by calculating the difference between the amount of protein applied and that remaining free in solution after the immobilization process. But very often inconsistent results are obtained, so that this method can only be used for obtaining a rough estimate. The gel-bound protein in the derivatives was measured directly, which is a great advantage, since methods are not always available for the determination of immobilized protein, without any interference from the matrix, yet at the same time being able to detect low protein content.

The direct measurement of gel-bound activity allows the calculation of the amount of immobilized enzyme which is present, and active, on the gel.

It is well known that an enzyme does not necessarily maintain intact its whole activity after immobilization, and that enzyme immobilization is not always synonymous with enzyme stabilization. In fact, our CDAP-derivative exhibited no enzyme inactivation during immobilization, whereas with the CPC-derivative a dramatic decrease in both S.A. and V_{app} values were observed, with respect to those values for the soluble enzyme.

Covalent binding of the enzyme on to the matrix prevents enzyme release. However, the main disadvantage of covalent bonds is that generally the conditions used for the immobilization as well as the immobilization reaction per se, can damage the enzyme. Enzyme inactivation can be attributed to several causes: conditions of immobilization (pH, ionic strength), the distortion caused in the native structure of the enzyme due to the formation of covalent linkages to the matrix, the characteristics of the microenvironment generated around the enzyme during the coupling process, and other factors. It has been reported in the literature that the direct linear plot method can give an indication as to the extent of the diffusional effects in the support, by analyzing the median values of the intersection points that determines the Kapp values [15,20]. Fig. 4 shows the deviations observed for the soluble enzyme and for two CDAP-derivatives prepared with (i) the lowest enzyme load assayed and (ii) the highest enzyme load assayed. The same graphical analysis was carried out for the CPC-derivative (data not shown). The deviations observed for the CDAP- and CPC-derivatives are not significant, in comparison with other deviations reported, for example, for the immobilization of β-galactosidase from *Aspergillus niger* on to a different porous carrier [15].

The immobilization conditions we used were the same for both derivatives, and cannot be responsible for the enzyme inactivation observed during immobilization on to the CPC-activated carrier as demonstrated by the results obtained for a parallel control using soluble enzyme, as discussed above. The covalent coupling reaction involving amino groups can occur either, via unipunctual or multipunctual interactions. They involve the amino terminal group of the enzyme or the ε-amino groups of lysine residues, and the reactive groups introduced on the supports. As the pH rises, the quantity of unprotonated amine groups on the protein surface increases, and these are the only ones able to produce a nucleophilic attack against the active groups in the support [21]. The pH (7.0) we used for the immobilization reaction was lower than the pK_a (10.0) of the ε-amino groups, thus, the protein amino groups involved in this immobilization were expected to be the amino terminal (pK_a between 7.6 and 8.0) [22]. Thus, the possibility that multipunctual interactions may be responsible for a putative distortion of the enzyme folding, is very unlikely. The enzyme inactivation we observed during immobilization on to the activated CPC-silica could

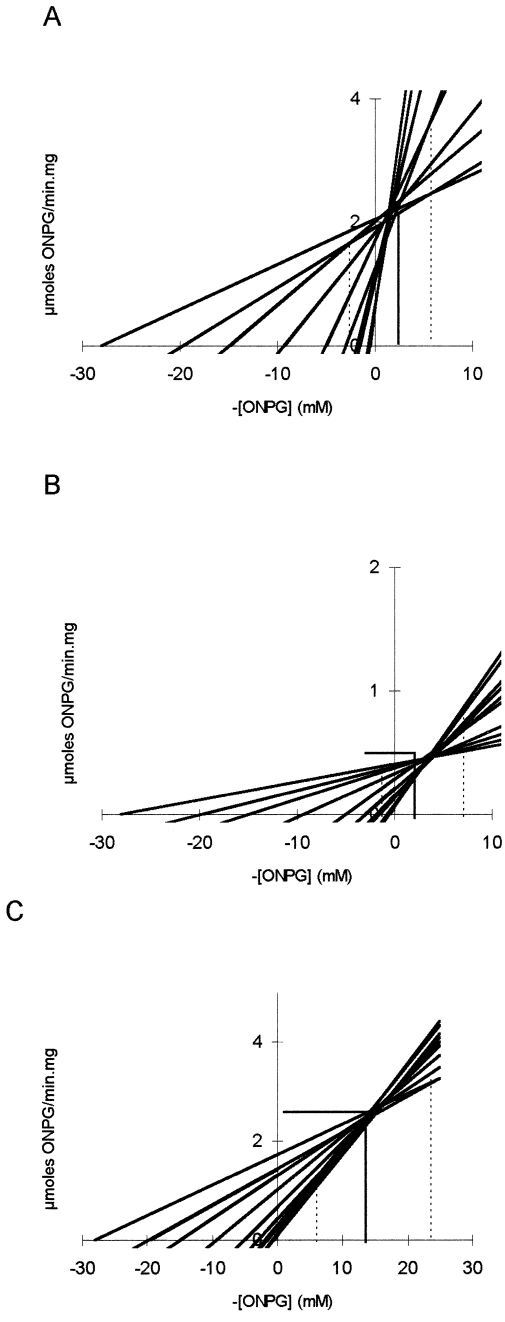

Fig. 4. Direct linear plot. (A) β-galactosidase from *K. lactis*, soluble form. (B) CDAP-derivative (1.0 mg ml^{-1} packed gel). (C) CDAP-derivative (5.4 mg ml^{-1} packed gel).

be due to the use of the bifunctional reagent, the glutaraldehyde. Although the activation of amino carriers with glutaraldehyde is based on the idea that one aldehyde group reacts with the matrix forming a Schiff's base and the other remains available for the further reaction with the protein, the nature of this reaction is not fully understood. Proteins are bound irreversibly to the glutaraldehyde-treated carrier by a reaction presumably analogous to that occurring during intramolecular cross-linking with this reagent, which is reported that could be responsible of changes in the protein conformation with a resultant loss of activity [19,23].

When synthesizing an enzyme derivative, determination of the kinetic parameters at each enzyme load assayed and subsequent comparison with those of the soluble enzyme yields valuable information about the influence of immobilization on enzyme activity. The increases in the K_{app} values of the CPC- and CDAP-derivatives with respect to the K_M value for the soluble enzyme are not unusual and can be due to diffusional effects [24]. They can also be partly explained by steric factors caused by neighboring enzyme molecules that could hinder the access of the substrate to some of the active sites causing diffusional effects [25]. Similarly, K_{app} values for CDAP-derivatives are also increased by increasing the enzyme loads, with a five-fold difference between the minimal and maximal enzyme loads assayed on the gel. No such effect was observed in the case of the CPC-derivatives, when we analyzed their K_{app} values together with the corresponding quantities of gel-bound protein (Table 4).

With respect to thermal stabilities, the CDAP-derivative shows better stability than the CPC-derivative but in neither case was the thermal stability of the soluble enzyme enhanced. These results are not surprising; in fact, it has been reported by Melrose [26] that a study of fifty immobilized enzyme systems showed that thirty had greater, eight had less and twelve had similar effective life time and temperature stability that the free enzyme.

In view of the possible applications of the obtained derivatives into industrial processes, we attempted to analyze their performance for the hydrolysis of lactose using buffered lactose

solutions and whey permeates. The reactor productivity (P) was expressed as:

$$P = C \times F$$

where C = concentration (mg ml^{-1}) of glucose in the output of the reactor under steady state conditions and F = flow rate (ml h^{-1}).

In spite of the fact that both derivatives achieved excellent productivities when used in packed bed mini-reactors, P achieved with the CPC-derivative was 3.3-fold greater than P achieved with the CDAP-derivative. This difference is a direct consequence of the higher flow rates used with the CPC-derivative packed-bed reactor, and is also in agreement with the characteristics of inorganic supports, in which higher flow rates can be achieved than in the case of organic supports such as agarose.

The productivity was also expressed considering the amount of enzyme in each reactor (P'):

$$P' = \frac{C \times F}{E}$$

where E = total enzyme units in the packed-bed reactor.

In this case, P' for the CDAP-derivative was 1.8-fold higher (0.9 mg of glucose h^{-1} EU^{-1}) than P' for the CPC-derivative (0.5 mg of glucose h^{-1} EU^{-1}). This result demonstrates that the derivatives we used in these lactolysis experiments contained more enzymatic activity (determined using ONPG as substrate) than the minimum enzyme activity required to achieve these very high degrees of lactose conversion in the steady state conditions of our reactor. Therefore, the optimal enzyme loads for any given derivative should be determined when considering the economic aspects of the method.

After reuse for lactose hydrolysis, we found that only 25% of the initially applied enzyme activity remained on the CPC-derivative. However, this residual gel-bound enzyme activity (1071 EU) was still higher than the enzyme activity initially bound to the CDAP-derivative. Nevertheless, lactose conversion was not affected by this decrease of the enzyme units in CPC-derivative and P' was 0.6 mg of glucose h^{-1} EU^{-1}, thus, reinforcing our statement that enzyme overloading had occurred.

When lactolysis was performed with whey permeate as the substrate, using the CDAP-derivative packed bed reactor, the concentration of mineral salts may have affected the enzyme activity; the β-galactosidase from *K. lactis* is known to be inhibited by Ca^{2+} ions [27], which are present in the whey permeate. The gel was extensively washed before determining the enzyme activity remaining on the derivative, thereby removing the calcium ions. This may explain why, even though there was no significant loss of enzyme activity by calcium ions at the end of the process, enzyme inhibition nevertheless occurred during operation. Studies to evaluate the effect of this ion as well as of sanitization procedures on these columns, are in progress.

5. Conclusions

Two biocatalysts were obtained by immobilizing β-galactosidase from *K. lactis* on to different porous supports. High loads of immobilized enzyme can be achieved using either of the two matrices, as determined by a direct method for measuring immobilized protein, described above. These derivatives were stable in the operating conditions described, and performed extremely well in lactose conversion. In order to improve cost-effectiveness, derivatives intended for industrial use with the lowest enzyme loads which permit the maximum degree of lactose hydrolysis should be taken into account.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Research/International Development Bank (CON-

ICYT-BID Program, Project 92/065), Montevideo, Uruguay, and the International Program In Chemical Sciences (IPICS), Uppsala University, Sweden.

References

[1] Y. Pomeranz, Food Technol. 18 (5) (1964) 96.
[2] A. Axelsson, G. Zacchi, Appl. Biochem. Biotechnol. 24–25 (1990) 679.
[3] V. Gekas, M. Lopez-Leiva, Process Biochem., February (1985) 1.
[4] J.G. Zadow, in: J.G. Zadow (Ed.), Whey and Lactose Processing, Elsevier, London, 1992, 377.
[5] H.H. Weetall, Appl. Biochem. Biotechnol. 41 (1993) 157.
[6] J. Kohn, M. Wilcheck, Appl. Biochem. Biotechnol. 9 (1984) 285.
[7] G. Antoni, R. Presentini, P. Neri, Anal. Biochem. 129 (1983) 60.
[8] J. Carlsson, J.C. Janson, M. Sparrman, in: J.C. Janson, L. Ryden (Eds.), Protein Purification, VCH Publishers, New York, 1989, 275.
[9] P.K. Smith, R.I. Khron, G.F. 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.
[10] K. Sorensen, U. Brodbeck, Experientia 42 (1986) 161.
[11] M.G. Redinbaugh, R.B. Turley, Anal. Biochem. 177 (1986) 41.
[12] T.M. Stich, Anal. Biochem. 191 (1990) 343.
[13] B.M. Brena, L.G. Ryden, J. Porath, Biotechnol. Appl. Biochem. 19 (1994) 217.
[14] G. Irazoqui, F. Batista-Viera, Biotechnol. Tech. 8 (4) (1994) 233.
[15] J. Peter, J.E. Prenosil, J.R. Bourne, J. Chem. Tech. Biotechnol. 31 (1981) 705.
[16] S.E. Kakabalos, P.E. Tyllianakis, G.P. Evangelatos, D.S. Ithakissios, Appl. Biochem. Biotechnol. 56 (1996) 95.
[17] J.C. Janson, J.A. Jönsson, in: J.C. Janson, L. Ryden (Eds.), Protein Purification, VCH Publishers, New York, 1989, 40.
[18] R.F. Taylor, in: R.F. Taylor (Ed.), Protein Immobilization, New York, 1991, 139.
[19] J.M.S. Cabral, J.F. Kennedy, in: R.F. Taylor (Ed.), Protein Immobilization, Fundamental and Applications, New York, 1991, 73.
[20] A.T. Cornish-Bowden, in: A.T. Cornish-Bowden (Ed.), Fundamentals of Enzyme Kinetics, Portland Press, London, 1995, 297.
[21] R.M. Blanco, J.J. Calvete, J.M. Guisán, Enzyme Microb. Technol. 11 (1989) 353.
[22] S.S. Wong, in: S.S. Wong (Ed.), Chemistry of Protein Conjugation and Cross-linking, CRC Press, 1993, 13.
[23] W. Hartmeier, in: W. Hartmeier (Ed.), Immobilized Biocatalysts, Springer-Verlag, Berlin Heilderberg, 1988, 22.
[24] M.D. Trevan, in: M.D. Trevan (Ed.), Immobilized Enzymes, Wiley, 1980, 36.
[25] K.D. Caldwell, R. Axén, M. Bergwall, J. Porath, Biotechnol. Bioeng. 18 (1976) 1573.
[26] G.J.H. Melrose, Rev. Pure Appl. Chem. 21 (1971) 83.
[27] Maxilat LX 5000. Product specification, Gist-brocades (1992).