ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2000) 63–69

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Stabilization of a β-glucosidase from *Aspergillus niger* by binding to an amine agarose gel

Giovanni Spagna [a,*], Riccardo N. Barbagallo [a], Pier Giorgio Pifferi [b], Rosa Maria Blanco [c], Josè Manuel Guisan [c]

[a] *Biotechnology Group from the Department of Horticulture, Floriculture, Arboriculture and Agroindustrial Technology (DOFATA), University of Catania, Via S. Sofia 98, 95123 Catania, Italy*

[b] *Biotechnology Group, Chemistry and Food Technology Specialisation School, Department of Industrial Chemistry and Materials, University of Bologna, Viale del Risorgimento 4, 40136 Bologna, Italy*

[c] *Instituto de Catalisis, Universidad Autonoma Cantoblanco, C.S.I C., 28049 Madrid, Spain*

Received 10 February 2000; received in revised form 6 June 2000; accepted 6 June 2000

Abstract

β-D-glucopyranosidase (βG, EC 3.2.1.21) is an enzyme of considerable importance in food technology for increasing the aroma of wines, musts, fruit juices and alcoholic beverages. In this research we have studied the stabilization of a commercial βG preparation, by covalent immobilization of its carbohydrate moiety to an amine agarose gel. The findings showed total adsorption of the enzyme, previously purified [G. Spagna, D. Romagnoli, A. Martion, G. Bianchi, P.G. Pifferi, Enzyme Microb. Technol., 22 (1998) 298], on the matrix, its low reduction in activity and finally a high stabilization over time.

Keywords: β-glucosidase; Enzyme stabilization; Amine agarose gel

1. Introduction

β-D-glucopyranosidase (BG, EC 3.2.1.21), together with other glycosidases (α-L-arabinofuranosidase, α-L-rhamnopyranosidase and β-D-apiosidase), is capable of hydrolysing the glycosides of monoterpenes, C13-norisoprenoids, sesquiterpenes and other aliphatic and aromatic alcohols, which are amongst the main components in the aroma of musts, wines [2–14] and fruit juices [15–19]. This enzyme is also capable of hydrolysing anthocyanins, which are the main colouring agents to be found in foods of vegetable origin. Moreover, by reversing the normal hydrolytic reaction, βG can also potentially be used in the synthesis of glycoconjugates [20,21]. The main interest in this enzyme is therefore related to its possible applications in beverage technology (such as in wine and fruit juice production) for the purpose of improving the sensorial properties of these products, such as aroma and colour [22–25]. In addition to the advantages already reported for the immobilization of other enzymes, the use of βG in its immobilized form [26–32] rather than in solution,

* Corresponding author. Tel.: +39-095-7142255; fax: +39-095-7141960.

E-mail address: spggnn@iol.it (G. Spagna).

1381-1177/00/$ - see front matter
PII: S1381-1177(00)00188-0

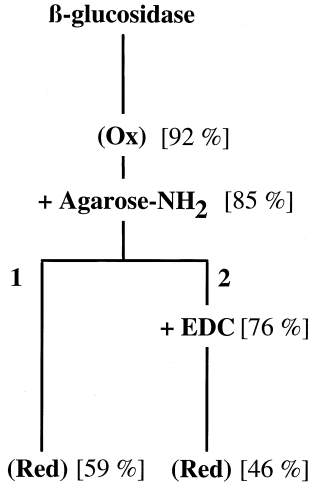

Fig. 1. Method and immobilization yields (IY, between parentheses) for immobilized βG on amine agarose gel.

may permit a better control of the reaction and, as its direct use in oenology is as yet not contemplated by European law, could also find wider application.

βG is a glycoenzyme featuring carbohydrate side chains [33], which, on the basis of our trials, do not appear to be directly involved in catalysis. In fact, after oxidation with periodate, the activity of the enzyme remains practically unchanged. Starting from these experimental findings and from those of similar studies by other authors [34], it was decided to immobilize this enzyme through its carbohydrate chains on a support containing amine groups. βG was thus immobilized on amine agarose gel as shown in Fig. 1. The method entails the oxidation of the carbohydrate chains of the enzyme, so as to form aldehyde groups capable of reacting with the amine groups of the matrix and their subsequent reduction. Moreover, before the reduction, the formation of covalent bonds with carbodiimide was also tested.

2. Materials and methods

2.1. Materials

β-glucosidase (βG) from *Aspergillus niger* was purified from the commercial preparation Cytolase PCL5 (PCL5, lot 44, Genencor, USA) by adsorption on bentonite (BDH, England). The matrix used for immobilization was crosslinked 6% agarose gel (Hispanagar S.A., Spain). The substrate employed to measure enzyme activity was 4-nitro-phenyl D-glucopyranoside (*p*-NPG); the other reagents: 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide (EDC), ethylene diamine dihydrochloride (FDA), glycidol, sodium periodate, sodium borohydride and trimethyl amino borane (TAB) were supplied from Sigma (St. Louis, USA).

2.2. Preparation of the amine agarose gel

The matrix was prepared according to Blanco et al. [35]. The method shown in Fig. 2 essentially comprises two steps. In the first step (a), agarose gel is transformed into the aldehyde derivative by etherification of the hydroxyl groups with glycidol and by oxidation of the resulting glyceryl-agarose with periodate. In the second step (b), the amine derivative is obtained from the matrix by functionalising the aldehyde groups with EDA and subsequently reducing them with $NaBH_4$.

1. Fifty millilitres of 28.5 g/l of $NaBH_4$ in 1.7 M NaOH solution and, very slowly, 36 ml of glycidol were added to 150 ml of agarose gel suspended in 30 ml of distilled water, under slow stirring. The mixture was allowed to react for about 18 h, closely checking that the temperature did not exceed 25°C. 150 ml of 0.1 M $NaIO_4$ solution were then added to the gel washed with abundant distilled water (about 1.5 l). The mixture was left to react under stirring at room temperature for about 2 h, the aldehyde derivative was thus obtained.
2. Thirty-five millilitres of this matrix were suspended in 200 ml of 0.2 M of EDA, which had been brought to pH 10.0 with 1 M HCl, and stirred at room temperature for 2 h. Two grams of $NaBH_4$ were then added and the suspension was stirred again for another 2 h. The resulting amine agarose gel was washed with 1 l of 0.1 M NaCl dissolved in 0.1 M borate buffer at pH 9.0 and in 0.1 M acetate buffer at pH 4.0, and finally with 1 l of distilled water.

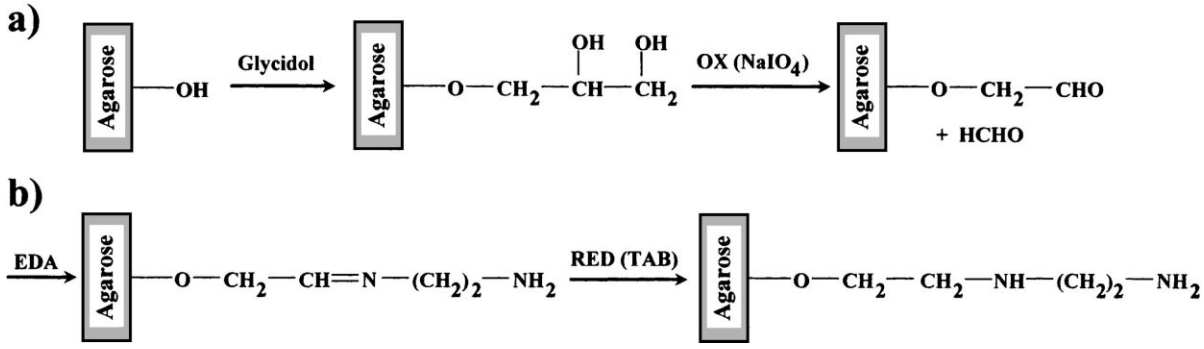

Fig. 2. Preparation of amine agarose gel.

2.3. Enzyme purification

This method is a modification of the one described by Martino et al. [1,36]. Ten grams of bentonite were slowly added to 250 ml of 0.1 M citrate-phosphate (C-P) buffer at pH 5.0. As soon as the dispersion became homogeneous, the pH was adjusted to 5.0 with 0.1 M NaOH. Two hundred and fifty millilitres of PCL5, which had also been brought to pH 5.0, were then added. The mixture was shaken for about 2 h, after which it was centrifuged at $10{,}000 \times g$ for 1 h. Fifty millilitres of 0.1 M C-P buffer at pH 5.0 containing a further 10 g of bentonite were added to the supernatant, after which the procedure was repeated as described above. To the enzyme solution, brought to pH 3.4 with 0.1 M HCl, were added 0.8 vol. of 0.1 M C-P buffer at pH 5.0, 0.2 vol. of ethanol and KCl up to about 0.25 M. A gelatinous precipitate was found in the solution after it had been left standing overnight at 3°C. This was removed by centrifugation at $10{,}000 \times g$ for 1 h at 3°C. The supernatant was brought to pH 7.5 with 0.1 M NaOH and ethanol was added up to 20%. As further impurities were seen to precipitate in the solution after it had been left to stand for 3 h at 30°C, centrifugation was once again performed so as to eliminate them. Amine gel was added to the solution brought to pH 6.0 with 0.1 M HCl in a 1:7 ratio (v/v). After filtration, the solution was ultrafiltered through a membrane with a molecular weight cut off of 30,000 (PM 30, Amicon). Finally, the enzyme was brought to pH 5.0 with a 0.05 M C-P buffer. This procedure was evaluated by determining βG activity, protein concentration [37], and brown compounds content measuring the decrease in absorbance at 420 nm.

2.4. Enzyme immobilization

Sixteen millilitres of 0.1 M $NaIO_4$ were added under stirring to 60 ml of purified enzyme (1.0–1.5 U ml^{-1}) brought to pH 5.0 with diluted HCl, after which the solution was allowed to react for about 2 h at 25°C. The enzyme solution was ultrafiltrated through a membrane with a molecular weight cut-off of 30,000. About 50 ml of 0.05 M C-P buffer at pH 5.0 were added after the enzyme had been washed with distilled water. Next, 2 ml of amine gel were added to 25 ml of the oxidized enzyme which had been brought to pH 5.5–6.0 and the enzyme was left to react under slow stirring for 2 h at room temperature. The suspension thus obtained was divided into two equal quantities, which were treated according to pathways 1 and 2.

In pathway 1, the suspension was allowed to stand overnight under stirring at 3°C. The gel was then filtered, washed with 0.05 M C-P buffer at pH 6.0 and reduced with 10 ml of 150 mM TAB in 0.05 M phosphate buffer at pH 8.0 for about 3 h.

In pathway 2, solid EDC was added up to 0.02 M to the suspension. After 2 h stirring at room temperature, the suspension was filtered, and 10 ml of TAB solution at pH 8.0 being added to the gel as in pathway 1.

2.5. Determination of enzyme activity

Depending on whether the enzyme was free or immobilized, 0.1 ml of a solution or a suspension of

enzyme, was added to 0.9 ml of 5.5 mM *p*-NPG dissolved in 0.1 M C-P buffer at pH 4.0. The assay was carried out under stirring at 25°C for 1 mm, after which adding 2 ml of 1 M Na_2CO_3 stopped it. The order of the reagents was reversed, for the blank test, i.e., the Na_2CO_3 solution was added to the substrate before the enzyme. The *p*-nitrophenol released in solution was measured at 400 nm, 18,300 M^{-1} cm^{-1} was adopted as the molar extinction coefficient. One activity unit of βG was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under assay conditions [38]. Finally, protein determination was carried out by colorimetric reaction with Coomassie Blue G250 according to Bradford [37].

Residual βG activity, at pH 4.0 (0.05 M C-P buffer) and 53°C, optimal pH between 3.0 and 7.5 (0.05 M C-P buffer) at 25°C, and finally an optimal temperature between 20 and 90°C at pH 4.0 (0.05 M C-P buffer) were also determined.

Adsorption yield (AY) and immobilization yield (IY) are defined as follows: $AY = U_{ads}/U_{eq} \times 100$; $IY = U_{act}/U_{ads} \times 100$; where U_{eq} are the enzyme units which are equilibrated with the support, U_{ads} are those which remain bound to the support after separation of the supernatant, and U_{act} are those which are active in the immobilized enzyme.

3. Results and discussion

The purification process adopted permitted a good recovery of enzyme activity (about 60%), with a five times increase in specific activity (from 1.4 to 7.1 U/mg of protein), a reduction of polysaccharides (ca. 15%) especially by precipitation, and a reduction in brown compounds (of at least 85%), resulting from Maillard's reaction during sterilization and fermentation of enzyme production. Enzyme purification was necessary, as the use of the commercial enzyme preparation as such did not permit the βG to be adsorbed on to the amine gel, as brown compounds compete with the enzyme for adsorption. This method of purification allowed total βG adsorption. Moreover, βG activity in the purified enzyme preparation was seen to remain almost unchanged (about 90%) after oxidation with periodate. This finding would seem to confirm the hypothesis that the side sugar chains are not involved in the catalysis. The adopted method and the immobilization yield values of the single steps are summarized in Fig. 1.

The enzyme was completely absorbed on amine gel (adsorption yield, AY 100%), maintaining in both pathways a considerable activity (immobilization yield, IY 59% and 46% for pathway 1 and 2, respectively). The oxidation of the enzyme before its immobilization on agarose-NH_2 permitted a good retention in activity (IY 85%). The addition of carbodiimide (EDC) gave only a slight decrease in immobilization yield. The main reactions which maybe involved are shown in Fig. 3. The first reaction involved is the oxidation of the sugar chains bound to the enzyme, with consequent formation of aldehyde groups [39,40]. These groups are capable of reacting with the amine groups (Schiff bases) present on the surface of the matrix, and probably also on that of the enzyme itself, via inter- and intra-molecular reactions. In pathway 1, the double bonds of the Schiff bases are reduced to single bonds, and the aldehyde groups, if any are free, are reduced to alcoholic ones. The addition of EDC in pathway 2 permits the formation of additional covalent bonds between the free amine groups of the support and the carboxylic ones of the enzyme [41]; this does not exclude other collateral reactions, such as cross-linking [42,43]. Such a model permits several hypotheses to be put forward for the interpretation of the previous results.

The enzyme molecules tend to orient themselves with the oxidized side sugar chains facing towards the surface of the matrix, in such a way as to favour the formation of the Schiff bases. This could 'freeze' the enzyme molecules in a favourable orientation with the carbohydrate chains that act as 'pads' (spacer arm), protecting the enzyme protein structure from conformational distortions induced by contact with the support [44,45].

These hypotheses seem to be confirmed by the lower immobilization yield (60%) obtained by the direct adsorption of the non-oxidized enzyme on the aminate agarose. In this case the protective shield effect given by the sugar chains could be reduced [46–48]. The immobilization was guided by the electrostatic interactions between the negatively charged

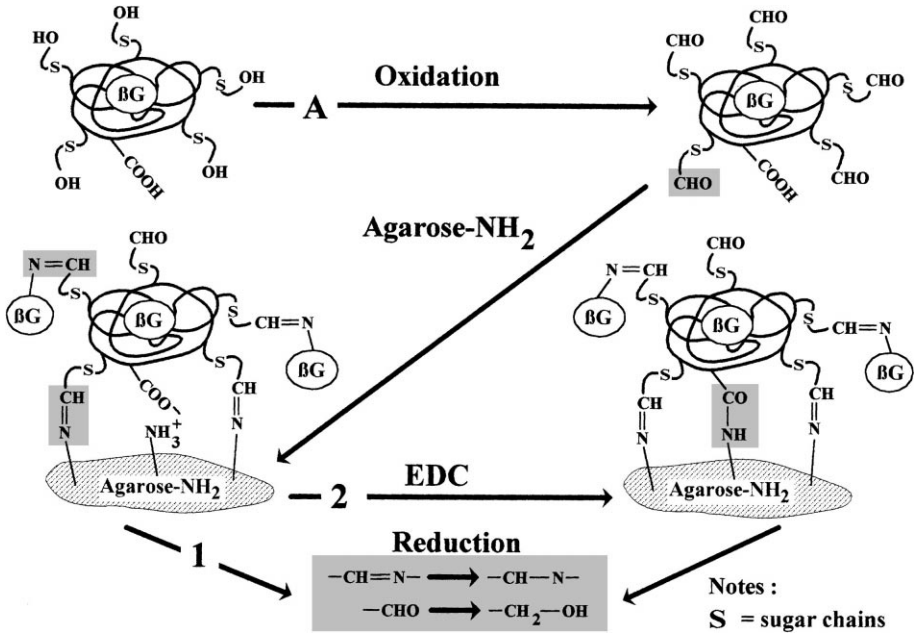

Fig. 3. Immobilization of βG according to method A and pathways 1 and 2. Main reactions are enclosed in boxes.

enzyme (pI 4.0) [46] and the positively charged amine gel (pK_a = 6.3) with a possible involvement of the carboxylic groups of the active site [49].

The addition of EDC in immobilization pathway 2 (Fig. 3), increases the number of covalent bonds for each immobilized enzyme molecule, thus causing an increase in the rigidity of the secondary and tertiary structure of the enzyme protein. This could lead to

1. involvement of the carboxylic groups of the enzyme active site, reduction in the enzyme diffusion rate, reduction in the conformational adaptability to the substrate [49], and consequently in immobilization yields;
2. a decrease in the irreversible unfolding rate and consequently, a simultaneous increase in stability [50–52].

As compared to the free enzyme, the immobilized β-glucosidase show

1. an optimum pH lower than 0.5, with almost the same trends for both immobilized enzymes (Fig. 4). The presence of aminic and amidic groups on the surface of the biocatalyst probably causes an increase in the concentration of OH^- in the enzyme microenvironment. The enzyme is therefore affected by a pH higher than that of the surrounding solution [53];
2. an optimum temperature of 72°C and 75°C for pathways 1 and 2 respectively, compared to that

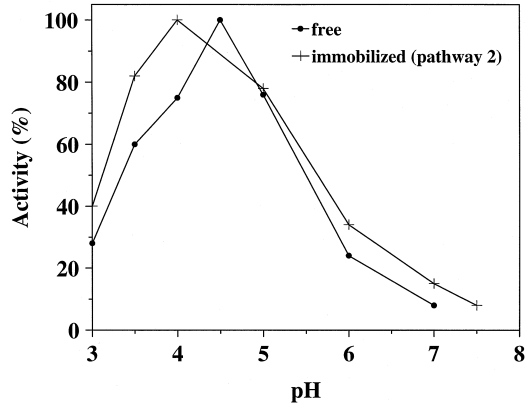

Fig. 4. Activity of the free and immobilized βG (pathway 2) as function of pH at 25°C.

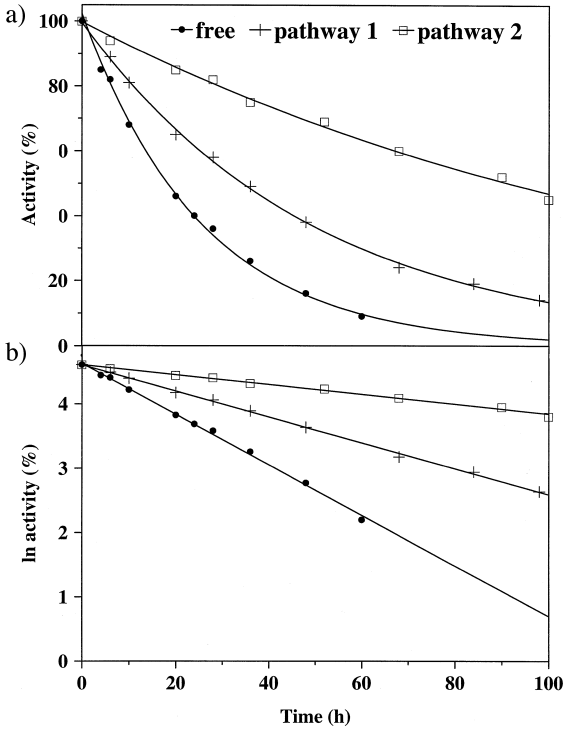

Fig. 5. Stability of the free and immobilized βG method, as relative (a) and logarithmic (b) activity at pH 4.0 and 53°C.

of 70°C for the free enzyme. The covalent and non-covalent bonds between the enzyme and the matrix may, in fact, reduce the degrees of freedom of the protein molecular structure of the enzyme, thus protecting it from denaturation caused by high temperature [53];

3. an increase in stability values, with a good increase in residual activities (22% after 24 h of storage) for pathway 2 in comparison to pathway 1 (Fig. 5a). From the linear plot of the stability data (Fig. 5b) the following increasing order for the inactivation constants (K_{in}) were determinate: pathway 2 ($7.6 \times 10^{-3}\ h^{-1}$), pathway 1($2.0 \times 10^{-2}\ h^{-1}$), and free enzyme ($3.9 \times 10^{-2}\ h^{-1}$). Particularly pathway 2, with the addition of EDC, stabilized better βG. Probably higher density of bonds between the enzyme and the support, due to the crosslinking reactions, reduces the degrees of freedom of the enzyme conformation and consequently the unfolding rate.

4. Conclusions

β-glucosidase immobilization via oxidation of its carbohydrate chains and reaction on amine agarose gel appears to be a suitable technique; in fact, the immobilized enzyme exhibits quite high immobilization yields and residual activity levels.

Further activation of the biocatalyst with carbodiimide, which increases the density of the covalent bonds for each immobilized enzyme molecule, only slightly affects activity, while considerably enhancing its stability over time.

In our opinion, this immobilization methodology could be tested on other glycosidases, such as α-L-arabinofuranosidase and α-L -rhamnopyranosidase, that contain carbohydrate chains [54–58].

References

[1] G. Spagna, D. Romagnoli, A. Martino, G. Bianchi, P.G. Pifferi, Enzyme Microb. Technol. 22 (1998) 298.
[2] E. Stahl Biskup, F. Intert, J.H. Holthnujzen, M. Stengele, G. Schultz, Flav. Fragr. J. 8 (1993) 61.
[3] J.W. Patriks, C.R. Strauss, W. Bevan, Phytochem. 21 (1981) 2013.
[4] P.J. Williams, C.R. Strauss, B. Wilson, R.A. Massy-Westropp, Phytochem. 21 (1982) 2013.
[5] K.G. Haight, B.H. Gump, Am. J. Enol. Vitic. 45 (1984) 113.
[6] C.R. Strauss, W. Bevan, P.R. Gooley, J.W. Patrik, Am. Chem. Soc. 4 (1986) 223.
[7] Z. Gunata, R. Bitteur, J.M. Brillouet, C. Bayonove, R. Cordonnier, Carbohydr. Res. 184 (1988) 139.
[8] R.E. Cordonneir, Y.Z. Gunata, R.L. Baumes, C.L. Bayonove, J. Internationale des Science de la Vigne et du Vin. 23 (1989) 7.
[9] S.G. Vorin, R.L. Baumes, S.M. Bitteur, Z.Y. Gunata, C.L. Bayonove, J. Agric. Food Chem. 38 (1990) 1373.
[10] P. Wu, M.-C. Kuo, T.G. Hartman, R.T. Rosen, C.-T. Ho, J. Agric. Food Chem. 39 (1991) 170.
[11] P.J. Williams, M.A. Sefton, I.L. Francis, Glycosidic precursors of varietal grape and wine flavor, Flavor Precursors, ACS Symp. Series 490 (1992) 74.
[12] C. Bayonove, Y.Z. Gunata, J.C. Sapis, R.L. Baumes, I. Dugelay, C. Grassi, Vignevini 9 (1993) 33.
[13] M.-P. Le Traon-Masson, P. Pellerin, Enzyme Microb. Technol. 22 (1998) 374.
[14] Y. Gueguen, P. Chemardin, G. Janbon, A. Arnaud, P. Galzy, Use of beta-glucosidase in the development of flavor in wines and fruit juices. Humana Press, New York, 1999, p. 7.
[15] W. Schawab, P. Schreier, J. Agric. Food Chem. 38 (1990) 757.

[16] W. Schawb, G. Scheller, P. Schreier, Phytochemistry 29 (1990) 607.
[17] P. Winterhalter, J. Agric. Food Chem. 38 (1990) 452.
[18] G. Krammer, P. Winterhalter, M. Schawb, P. Schreier, J. Agric. Food Chem. 39 (1991) 778.
[19] C. Marlatt, C.-T. Ho, M. Chien, J. Agric. Food Chem. 40 (1992) 249.
[20] G. Nicolini, F. Mattivi, G. Versini, Riv. Vitic. Enol. 1 (1994) 45.
[21] C.L. Gunata, C.T. Baynove, R.E. Tapiero, R. Cordonnier, J. Agric. Food Chem. 38 (1990) 1232.
[22] A.P. Aryon, B. Wilson, C.R. Strauss, P.J. Williams, Am. J. Enol. Vitic. 38 (1987) 182.
[23] H. Bloom, M.S. Thomassen, Food Chem. 17 (1985) 1567.
[24] G. Vic, D. Thomas, Tetrahedron Lett. 33 (1992) 4567.
[25] C.T. Ravet, M.D. Legoy, Biotech. Bioeng. 42 (1993) 303.
[26] O. Shoseyov, B.A. Bravdo, D. Siegel, A. Goldman, S. Cohen, L. Shoseyov, R. Ikan, J. Agric. Food. 38 (1990) 1387.
[27] J.L. Iborra, M.R. Castellar, M. Canovas, A. Manjon, Enzyme Microb. Technol. 15 (1993) 780.
[28] A.C. Chakrabarti, K.B. Storey, Appl. Biochem. Biotech. 22 (1989) 263.
[29] M.D. Fadda, M.R. Dessi, A. Rinaldi, G. Satta, Biotech. Bioeng. 33 (1989) 777.
[30] J.R. Woodward, A. Radford, L. Rodriguez, J. Aguado, M.D. Romero, Acta Biotech. 12 (1992) 357.
[31] Y. Vasserot, A. Arnaud, P. Galzy, Bioresour. Technol. 43 (1993) 269.
[32] V.V. Mozhaev, N.S. Melik-Nubarov, V. Siksnis, K. Martinek, Biocatalysis 3 (1990) 189.
[33] M.E. Himmel, W.S. Adney, J.M. Fox, D.J. Mitchell, J.O. Baker, Appl. Biochem. Biotech. 39/40 (1993) 213.
[34] M. Marek, O. Valentovà, J. Kas, Biotech. Bioeng. 26 (1984) 1223.
[35] R.M. Blanco, J.M. Guisán, Enzyme Microb. Technol. 10 (1988) 227.
[36] A. Martino, P.G. Pifferi, G. Spagna, J. Chem. Tech. Biotech. 61 (1994) 255.
[37] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[38] C. Romero, A. Marjòn, L. Bastida, J.L. Iborra, Anal. Biochem. 149 (1985) 566.
[39] B. Kozulic, I. Leustek, B. Pavlovic, P. Mildner, S. Barbaric, Appl. Biochem. Biotech. 15 (1987) 265.
[40] J.M. Guisán, Enzyme Microb. Technol. 15 (1993) 1.
[41] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida, R. Timkovich, Biochem. Biophys. Res. Comm. 74 (1977) 1463.
[42] A.V. Papisov, A.V. Maksimenko, V.P. Torchilin, Enzyme Microb. Technol. 7 (1985) 11.
[43] A. Tsutomu, S. Timasheff, Biochemistry 21 (1982) 6536.
[44] J.M. Guisán, Enzyme Microb. Technol. 10 (1988) 375.
[45] K.N. Neustroev, A.M. Golubev, L.M. Firstov, F.M. Ibatullin, I.I. Protasevich, A.A. Makarov, FEBS Lett. 316 (1993) 157.
[46] M. Wahlgreen, T. Arnebrant, Tibtech. 9 (1991) 201.
[47] W. Norde, Adv. Coll. Interface Sci. 25 (1986) 267.
[48] T.A. Horbett, J.L. Brash, Proteins at interfaces: current issues and future prospects, in: J.L. Brash, A.T. Horbett (Eds.), Proteins at Interfaces, ACS Symp. Series, 1987, p. 1.
[49] T. Watanabe, T. Sato, S. Yoshioka, Eur. J. Biochem. 209 (1992) 651.
[50] R.M. Blanco, J.M. Guisán, Enzyme Microb. Technol. 11 (1989) 360.
[51] V.V. Mozhaev, K. Martinek, Adv. Drug Deliv. Rev. 4 (1990) 387.
[52] V.V. Mozhaev, Tibtech. 11 (1993) 88.
[53] I. Chibata, Properties of immobilized enzymes and microbial cells, in: I. Chibata (Ed.), Immobilized Enzymes, Vol.3, Halsted Press, New York, 1978, p. 108.
[54] A. Kaji, Adv. Carbohydr. Chem. Biochem. 42 (1984) 383.
[55] Z. Gunata, J.M. Brillout, S. Vorin, R. Baumes, R. Cardonnier, J. Agric. Food Chem. 38 (1990) 772.
[56] S. Kaneko, T. Shimasaki, I. Kusakabe, Biosci. Biotech. Biochem. 57 (1993) 1161.
[57] M. Roitner, T. Schalkhammer, F. Pittner, Monatshefte für Chemie 12 (1984) 1255.
[58] C. Romero, A. Manjòn, L. Bastida, J.L. lborra, Anal. Biochem. 149 (1985) 566.