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Food Chemistry 97 (2006) 591–597
Food Chemistry
www.elsevier.com/locate/foodchem

Polyacrylamide–gelatine carrier system used for invertase immobilization

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Received 24 September 2004; received in revised form 18 February 2005; accepted 19 May 2005

Abstract

Invertase was immobilized into polyacrylamide–gelatin carrier system by chemical cross-linking with chromium (III) acetate, chromium (III) sulphate, and potassium chromium (III) sulphate. The optimum conditions, namely substrate concentration, temperature, and pH were determined. The effect of polyacrylamide–gelatin ratio and cross-linker concentration on immobilized enzyme activity were analysed. Maximum immobilized enzyme activities were obtained with chromium (III) acetate (0.01 mol dm^{-3}), chromium (III) sulphate (0.004 mol dm^{-3}) and potassium chromium (III) sulphate (0.001 mol dm^{-3}) for 0.177 (w/w) polyacrylamide–gelatin carrier ratio as 79%, 72% and 79%, respectively. The K_m values were 86 and 166 mM for free and immobilized enzyme, respectively. All immobilized samples were used 20 times over a period of 2 months without a considerable loss of activity.
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Keywords: Gelatin; Polyacrylamide; Invertase enzyme; Immobilization

1. Introduction

Enzymes are biological catalysts that increase the rate of chemical reactions taking place within living cells by lowering the activation energy. Immobilization of enzyme is undertaken either for the purpose of basic research or for use in technical processes of commercial interests (Cirpan, Alkan, Toppare, Hepuzer, & Yagci, 2003; Danisman, Tan, Kacar, & Ergene, 2004; Erginer, Toppare, Alkan, & Bakir, 2000).

Many methods have been described for the attachment of enzymes to supporting structures. The activity of an immobilized enzyme depends on a number of factors which can be summarized as size and shape of the carrier material, the nature of the immobilization method, the composition of the carrier material and the specific conditions during immobilization. The success of an immobilized enzyme for practical applications depends strongly on the properties of the carriers employed. The carrier should have a high capacity for forming large number of bonds such that each unit of carrier can immobilize large amounts of enzymes, if desired. Carriers should also be chemically stable and inert towards microbiological contamination so that they do not deteriorate due to attack by microorganisms and should thereby provide an immobilized enzyme system having a prolonged life of activity.

A large variety of carrier materials which may be organic or inorganic in structure, have been used for the attachment of enzymes (Coughlin & Charles, 1980; Zaborsky, 1973). Among the carriers, gelatin (G) is a widely used natural polymer which is a product of partial hydrolysis of collagen. Reactive groups present in

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0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.05.017

gelatin are primarily hydroxyl, carboxyl, and amino functions. Their abondances are approximately 100, 75 and 30 milliequivalents (per 100 g of quality gelatin), respectively (Sungur & Akbulut, 1994; Yılıdrım, Akbulut, Arínç, & Sungur, 1994). Inorganic compounds can harden gelatin by chemical cross-linking. Among the inorganic hardeners alums and chromium salts are the most commonly used compounds. Hardening with alums and chromium salts occurs by the formation of cross-links between the carboxyl groups of the gelatin through their divalent metallic ions. Gelatin is known to harden with chromium (III) acetate (CA), chromium (III) sulphate (CS), and potassium chromium (III) sulphate (PCS) whereas polyacrylamide (PAA) on its own does not harden under the same experimental conditions (Kirk-Othmer, 1986). Polyacrylamide is a well-known high molecular weight polymer which has the advantage of good chemical stability, a uniform physical state, porosity, and commercial availability (Kirk-Othmer, 1986; Sungur, Elçin, & Akbulut, 1992).

Immobilization of invertase has reached a high level of performance which led to some important large scale applications. Among the industrial applications the most important one is the use of immobilized invertase in converting sucrose to glucose and fructose in food industry to prevent crystallization of sucrose in sugar mixtures. The product obtained by invertase has the advantage of being colourless compared to the coloured version obtained through acid hydrolysis (Marek, Valentino, & Kas, 1984; Kotzelski & Staude, 1996; Mansfeld, Forster, Schellenberger, & Dautzengenberg, 1991). It is widely used in the production of noncrystallizing creams, for making jams and is also used in the production of artificial honey, and to a small extent in the industrial production of liquid sugar (Hartmeimer, 1996). Many studies were performed on the immobilization of invertase on microporous pHEMA-GMA membrane (Danisman et al., 2004), *Cajanus cajan* lectin support (Ahmad, Anwar, & Saleemuddin, 2001), poly(aniline-acrylic acid) film (Chen, Kang, Neoh, & Tan, 2000), dimmer acid-co-alkyl polyamine granules (Tümtürk, Arslan, Disli, & Tufan, 2000), acrylamide/maleic acid cylinder (Arslan, Tümtürk, Caykara, Sen, & Güven, 2000), calcium alginate beads (Arruda & Vtole, 1999), materials such as anion exchanger wates cotton thread (Godbole, Kubal, & D'Souza, 1990), polyacrylonitrile (PAN) and carboxyl-modified polyacrylonitrile (PAN-AA) ultrafiltration membranes (Mathias & Alexandra, 1997), and carboxymethylcellulose-gelatin (Emregül, Sungur, & Akbulut, 1996). The invertase has been immobilized into conducting polymers by electropolymerization (Alkan, Toppare, Yagci, & Hepuzer, 1999; Cirpan, Alkan, Toppare, Yagci, & Hepuzer, 2002; Cirpan et al., 2003; Kizilyar, Akbulut, Ozden, Toppare, & Yagci, 1999; Selampinar, Akbulut, Ozden, & Toppare, 1997).

In the present work, cost, physiological inertness, structural suitability for immobilization was the criteria for choosing gelatin as carrier material. Polyacrylamide was used as filling material in order to increase the surface area of the carrier system. Invertase was immobilized by chemical cross-linking in carrier systems obtained from various ratios of PAA-G. The resultant immobilized invertase system was chracterized and its activity retention, catalytic properties, and reusability were determined.

2. Materials and methods

2.1. Materials

Invertase [EC 3.2.1.26] and sucrose were obtained from Sigma Chemical Co. [USA]. Polyacrylamide was supplied from Aldrich [USA] having an average molecular weight of about 5–6 million. Granular photographic gelatine was obtained from Croda Gelatine Co. [UK]

Polyester films pre-coated with photographic gelatine (100 μm) were obtained from Dupont De Nemours [Luxemburg]. Chemicals used in preparation of buffers and cross-linking agents were purchased from Merck [Germany].

2.2. Methods

2.2.1. Immobilization of invertase

Gelatin and polyacrylamide blends were prepared by dissolving them in EDTA buffer (pH 7.2, 0.1%) to obtain a final weight of 0.75 g polymer in final volume. Invertase solution (24 U) and appropriate amounts of cross-linkers were added to carrier system solutions at 32 °C to obtain final volume of 10 cm^3. The solutions were stirred for 1 min and 0.1 cm^3 aliquots were taken and placed on polyester film strips. The strips were dried at room temperature for 24 h. Enzyme leakage from immobilized film strips were controlled by washing with EDTA (pH 7.2, 0.1%)

2.2.2. Determination of invertase activity

Free and immobilized enzyme activities were determined according to the Nelson method (Sidney & Colowick Nathan, 1957). The reaction by free or immobilized enzyme were carried out in a mixture of free or immobilized enzyme, ETDA buffer (pH 7.2, 0.1%), acetate buffer (pH 4.6, 0.05 mol dm^{-3}). Following the preincubation of the reaction mixture (2 min, 25 °C), substrate solution (0.3 mol dm^{-3}, sucrose) was added and incubated for 10 min at the same temperature. The enzymatic process stopped by adding 1 cm^3 of Nelson Reagent. The activity was determined from the amount of reducing sugar produced.

The absorbencies were measured at 540 nm by using Spectronic 20 D Milton Roy model spectrophotometer. One unit of enzyme was defined as the quantity of enzyme which hydrolyses 1 μmol of sucrose to glucose and fructose per minute.

% Relative activities were calculated according to the following formula:

$$\mathrm{ra} = \frac{\text{activity of complex}}{\text{total activity of enzyme used for coupling} - \text{activity loss by enzyme leakage}},$$

ma : maximum value of ra in series of experiments,

% Maximum activity : ra $\times 100/\mathrm{ma}$.

3. Results and discussions

3.1. Effect of cross-linker concentrations

Invertase was immobilized into different carrier systems obtained by mixing with different ratios of PAAG (0.000–0.333 w/w) with cross-linkers CA (0.01–0.06 mol dm^{-3}) CS (0.004–0.036 mol dm^{-3}) and PCS (0.001–0.017 mol dm^{-3}). Enzyme leakage test were performed for all immobilized samples with EDTA buffer (pH 7.2, 0.1%) for 12 min at 25 °C.

The results of washing procedure of the film strips have shown that in PAA-G carrier systems the enzyme leakage is disregardingly low and could even be zero when the PAA-G ratio is increased.

Different concentrations of cross-linkers were used for enzyme immobilization onto carrier systems obtained by using different ratios of PAA-G. Results are given in Fig. 1. Increasing the cross-linker concentration decrease the relative activities of immobilized enzyme. This can be explained as inactivation of enzyme molecules and a more tight gel caused by the usage of excess cross-linker.

The inactivation of enzyme by cross-linker, due to the direct interaction of the free invertase with the cross-linker has been investigated. In free enzyme inactivation experiments both cross-linker and enzyme concentrations were kept the same as those in immobilized system. Results are presented in Fig. 2. The enzyme inactivation by cross-linkers has a more pronounced effect on activity decrease. Excess cross-linkers must have inactivated some of the enzyme molecules by blocking the active site of the enzyme (Emregül et al., 1996). The results showed the effect of enzyme inactivation to be more important for cross-linker CS, especially when it was used above 0.020 mol dm^{-3}.

3.2. Effect of weight ratios of PAA-G

We previously reported the immobilization of invertase onto gelatin and carboxymethylcellulose–gelatin carrier system with cross-linkers CA, CS and PCS. Relative activities obtained for a carrier system composed of pure gelatin with 0.01 mol dm^{-3} CA, 0.004 mol dm^{-3} CS and 0.001 mol dm^{-3} PCS were found to be 32%, 38%, 56%, respectively (Emregül et al., 1996). To achieve enhanced sucrose conversion to glucose and fructose, we examined the possibility of utilizing the PAA based amplification method to augment immobilized invertase on the gelatin–invertase systems for sucrose interactions. The effect of carrier composition on relative activity of immobilized invertase were determined for PAA-G ratios. Polyacrylamide was chosen as filling material because it does not harden when treated with cross-linkers (CA, CS or PCS) under predefined immobilization conditions. Different ratios of PAA-G (0.053–0.333 w/w) were tested and results are presented in Fig. 3.

As the ratio of PAA increased, the relative activity increase due to the enlarged surface area of gelatin upto 0.177 PAA-G, w/w, was obtained. The increase in the relative activity at low ratios must be associated with an increase in the amount of immobilized surface enzyme. Increasing the ratio of PAA to higher levels resulted in a decrease in enzyme activity. The decrease in the relative activity at high PAA ratios suggests that in the presence of diffusion limitations, a considerable fraction of the enzyme molecules are probably embedded in the polymer and becomes less accessible. The inhibition in enzyme activity at high PAA ratios can arise from the increase in steric hindrance, and increase in interaction between the active sites of the enzyme and the PAA. The increase of PAA content possibly caused higher levels of binding between PAA and the enzymes which resulted in poorer relative activity yields, probably because of excess enzyme binding, leading to inactivation (Emregül, Sungur, & Akbulut, 1995). PAA had a positive effect on relative activity for low ratios and negative effect for high ratios. Thus, the maximum relative activities were increased to 79%, 72%, and 79% for 0.177 PAA-G, w/w, carrier ratio by same cross-linker concentrations as in pure gelatin. A relative increase of 47% for CA, 34% for CS and 23% for PCS was accomplished by changing the ratio of carrier system. The best results were obtained for PAA-G ratio 0.177 (Fig. 3).

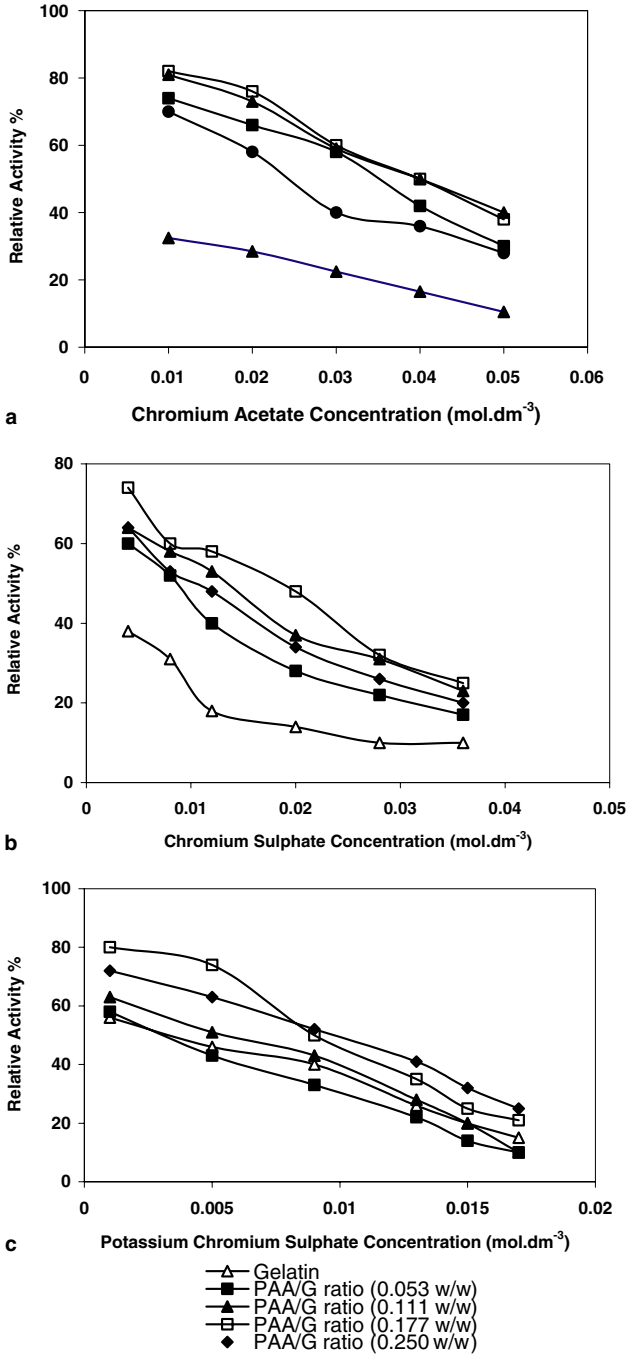

Fig. 1. Effect of cross-linker concentration on the activity of PAA-G-Invertase. Conditions for the preparation of PAA-G-Invertase: 0.177 w/w ratio PAA/G, (a) 0.01–0.06 mol dm^{-3} CA, (b) 0.004–0.036 mol dm^{-3} CS, (c) 0.001–0.017 mol dm^{-3} PCS, 0.3 mol dm^{-3} sucrose.

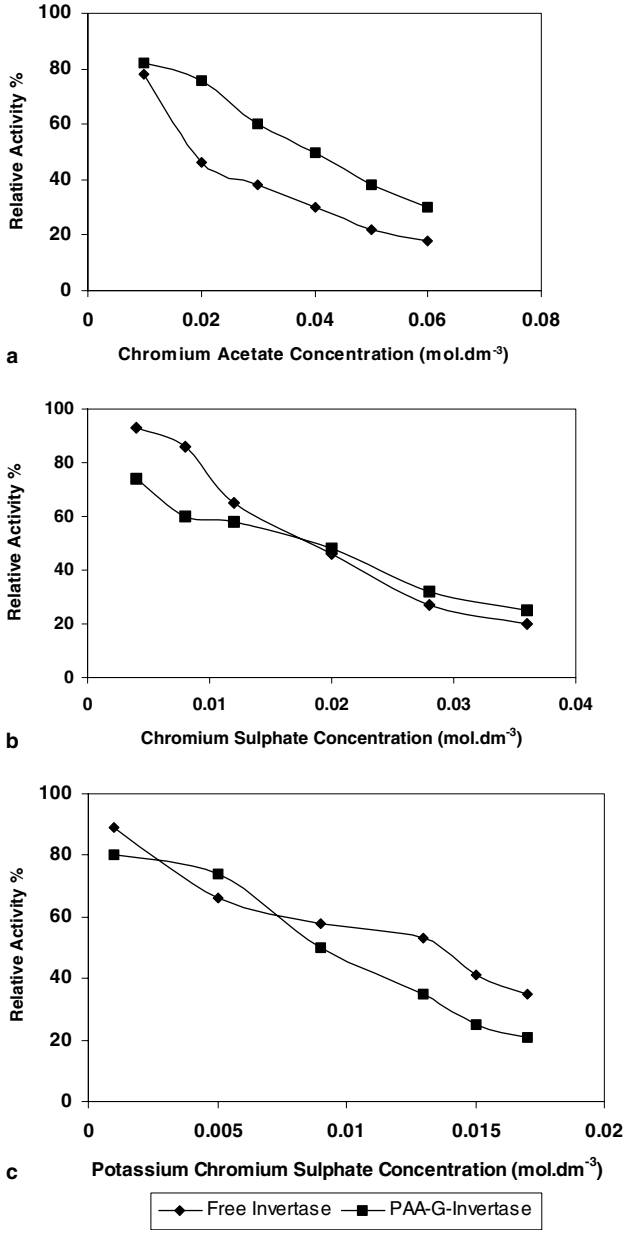

Fig. 2. Effect of cross-linker concentration on the activity of free invertase. Conditions for the preparation of PAA-G-Invertase: 0.177 w/w ratio PAA/G, (a) 0.01–0.017 mol dm^{-3} CA, (b) 0.004–0.036 mol dm^{-3} CS, (c) 0.001–0.017 mol dm^{-3} PCS, 0.3 mol dm^{-3} sucrose.

3.3. Temperature dependence

Effect of temperature variations on free enzyme and CA-immobilized invertase activities were investigated (Fig. 4). Reactions were carried out with 0.3 mol dm^{-3} sucrose concentration at pH 7.2 and the temperature influence was studied within the 5–70 °C range. The activities of free and immobilized enzyme were found to increase continuously with increasing temperature upto 55 °C then declined. The free and immobilized enzyme exhibited a temperature optimum of 55 °C. The activity of the invertase is strongly dependent on temperature. The activity of the free invertase showed a more critical temperature dependence compared to the immobilized invertase. The immobilized invertase retained 43% of its optimum activity whereas the free was 4% at 10 °C. The free and immobilized invertases retained 7% and 30% of their optimum

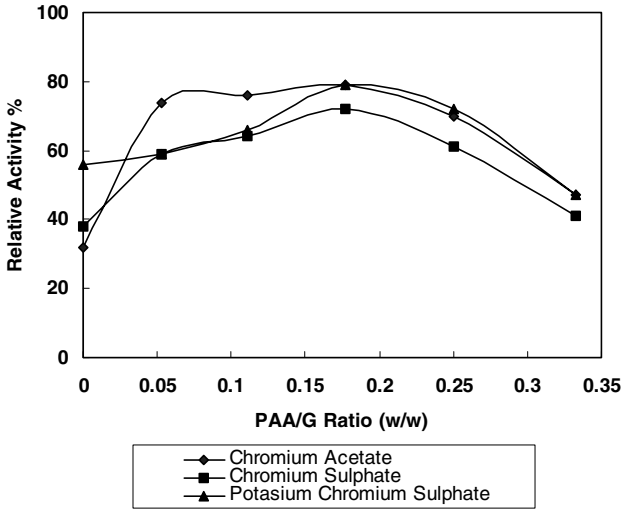

Fig. 3. Influence of PAA-G ratios on the activity of PAA-G-Invertase. Conditions for the preparation of PAA-G-Invertase: PAA/G ratios are 0.053, 0.111, 0.177, 0.250, w/w; 0.01 mol dm^{-3} CA, 0.004 mol dm^{-3} CS, 0.001 mol dm^{-3} PCS, 0.3 mol dm^{-3} sucrose.

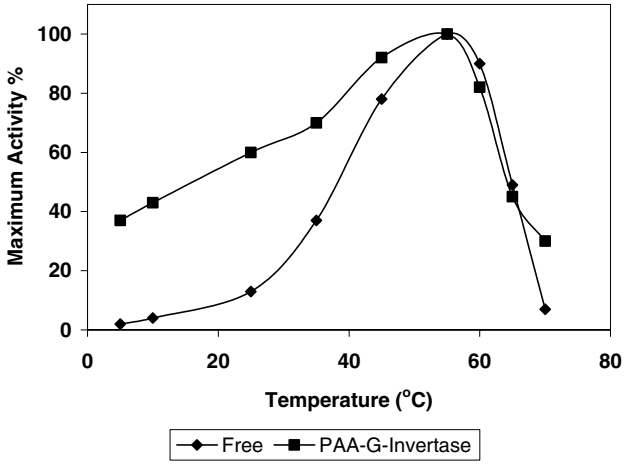

Fig. 4. Dependence of temperature on the activity of free and PAA-G-Invertase. Conditions for the preparation of PAA-G-Invertase: 0.177 w/w ratio PAA/G, 0.01 mol dm^{-3} CA; 0.3 mol dm^{-3} sucrose.

activities at 70 °C. The difference in the temperature activity profiles between the free and immobilized invertase is that the latter is less susceptible to temperature-induced conformational changes after immobilization into PAA-G system while the quarternary structure of the free invertase may be easily disaggregated (Esmon, Esmon, Schauer, Taylor, & Schekman, 1987; Greenfield & Laurence, 1975). The immobilization procedure probably helps to maintain the oligomeric forms (mainly octameric and hexameric aggregates) of the enzyme prevailing in the free invertase solution (Reddy, Maccoll, & Maley, 1990). The denaturation of the immobilized enzyme could take place on the tertiary structures of the peptidic chain of the invertase, which would also occur for the free form at 70 °C (Garnier, Youssoufi, Srivastava, & Yassar, 1994). During thermal experiments the loss of activity at higher temperatures can be attributed to the denaturation of some enzyme molecules, leaching of enzyme from the swollen polymer matrix and degradation of polymer matrix. The leakage of enzyme and the denaturation of gelatin does not constitute a major problem at 25 °C, which was selected as the incubation temperature throughout the work. The immobilized invertase displays significantly improved stability over the free form at lower and higher temperatures.

3.4. Effect of immobilization on enzyme kinetics

In order to study the kinetic effect of immobilization, the rates of sucrose hydrolysis reaction by the free and immobilized invertase were measured at various sucrose concentration (0.05–0.525 mol dm^{-3}). The Michaelis–Menten constant, K_m and maximum reaction rates, V_m were obtained from Lineweaver–Burke plot (Lineweaver & Burk, 1934). In this study, V_m values for free and immobilized invertase were found as 2.1 and 1.92 μmol min^{-1}, respectively. The Michaelis constants, K_m, of free and immobilized invertase were determined to be 86 and 166 mM, respectively.

K_m and V_m values were affected after immobilization of invertase. The K_m value of PAA-G-Invertase was approximately 1.92-fold higher than that of free enzyme. The difference in K_m values between the free and immobilized invertase can be attributed to the limited accessibility of sucrose molecules to the active sites of the immobilized invertase as a result of the spatial distribution of the invertase molecules in the polymer layer and conformational changes of invertase caused by the immobilization. The decrease in V_m value as a result of immobilization is considered to be associated with the external and internal diffusional resistances. This resistances can include external and internal transport of the substrate and products to the surface of the membrane (Danisman et al., 2004; Prodanovic, Javonovic, & Vujcic, 2001).

3.5. pH dependence

The free and immobilized immobilized invertase activities as a function of pH using 0.3 mol dm^{-3} sucrose were determined. The pH influence was studied within the 3.6–10 pH range. The results obtained are presented in Fig. 5. The optimum pH of free enzyme and PAA-Invertase was 6 and 7.2, respectively. The pH shift towards alkaline value upon immobilization is due to the secondary interactions between enzyme and the polymeric matrix (Arica, Denizli, Baran, & Hasirci, 1998; Erginer et al., 2000; Mansfeld et al., 1991; Tien & Chiang, 1999). The support is a charged

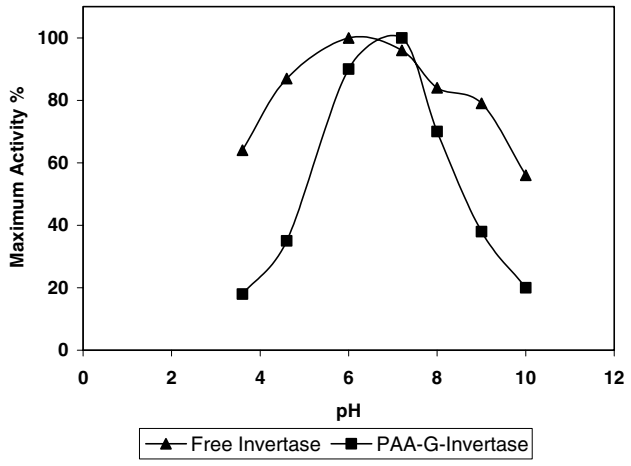

Fig. 5. Influence of pH on the activity of free and PAA-G-Invertase. Conditions for the preparation of PAA-G-Invertase: 0.177 w/w ratio PAA/G, 0.01 mol dm^{-3} CA; 0.3 mol dm^{-3} sucrose.

polymer which will affect the local pH. The polar groups of gelatin may have interacted with the functional groups of invertase changing the pH characteristics of the enzyme.

3.6. Reusability of PAA-G-invertase

Reusability of immobilized invertase was analysed by measuring activities of all samples 20 times over a period 2 months. Reusability tests were carried out for CA-, CS- and PCS- immobilized invertase for gelatin and 0.177 PAA-G ratio. Results are presented in Fig. 6. There was no significant difference in the relative activities over 20 times tested. Immobilized enzymes were durable and could be used at least 20 times without considerable activity loss.

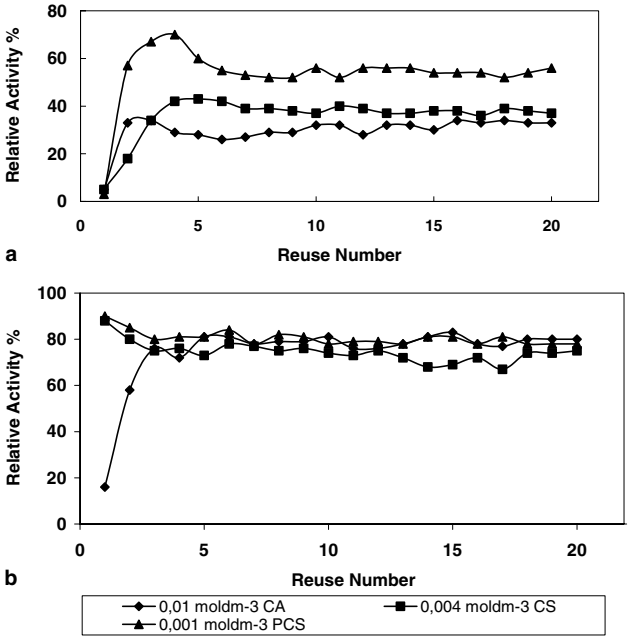

Fig. 6. Reusability of immobilized invertase with CA, CS, PCS in gelatin systems (a) and PAA-G systems (b). Conditions for the preparation of gelatin–invertase: 0.177 w/w ratio PAA/G, 0.01 mol dm^{-3} CA, 0.004 mol dm^{-3}, CS, 0.001 mol dm^{-3} PCS, 0.3 mol dm^{-3} sucrose.

4. Conclusion

Invertase enzyme was immobilized into PAA-G carrier systems with cross-linkers CA, CS and PCS. By using PAA; 47% for CA, 34% for CS and 23% for PCS relative activity enhancements were achieved. Immobilized samples obtained were stable and could be used many times over a period of 2 months without considerable activity loss. The pH value for optimum activity of invertase was not affected by the immobilization reaction. The temperature dependent activity profile for the immobilized invertase is broadened. The immobilized invertase displays significantly improved stability over the free form. These properties prove the usefulness of the examined materials in continuous reactors and biosensors in biotechnological applications.

References

Ahmad, S., Anwar, A., & Saleemuddin, M. (2001). Immobilization and stabilization of invertase on *Cajanus cajan* lectin support. *Bioresource Technology, 79*, 121.

Alkan, S., Toppare, L., Yagci, Y., & Hepuzer, Y. (1999). Immobilization of invertase in thiophene-capped poly (methy methacrylate)/polypyrrole matrices. *Journal of Biomaterials Science – Polymer Edition, 10*(12), 1223–1235.

Arica, M. Y., Denizli, A., Baran, T., & Hasirci, V. (1998). Dye derived and metal incorporated affinity poly(2-hydroxyethyl methacrylate) membranes for use in enzyme immobilization. *Polymer International, 46*, 345–352.

Arslan, F., Tümtürk, T., Caykara, T., Sen, M., & Güven, O. (2000). The effect of gel composition on the adsorption of invertase on poly(acrylamide/maleic acid) hydrogels. *Food Chemistry, 70*, 33–38.

Arruda, L. O. M., & Vtole, M. (1999). Characterization of invertase entrapped into calcium alginate beads. *Applications in Biochemistry and Biotechnology, 81*, 23.

Chen, Y., Kang, E. T., Neoh, K. G., & Tan, K. L. (2000). Covalent immobilization of invertase onto the surface-modified polyaniline from graft copolymerization with acrylic acid. *European Polymer Journal, 36*, 2095.

Cirpan, A., Alkan, S., Toppare, L., Yagci, Y., & Hepuzer, Y. (2002). Conducting graft copolymers of poly (3-methythienly methacrylate) pyrrole and thiophene. *Journal of Polymer Science – Polymer Chemistry, 40*, 4131–4140.

Cirpan, A., Alkan, S., Toppare, L., Hepuzer, Y., & Yagci, Y. (2003). Immobilization of invertase in conducting copolymers of 3-methylthienyl methacrylate. *Bioelectrochemistry, 59*, 29–33.

Coughlin, R. W., & Charles, M. (1980). In W. H. Pitcher (Ed.), *Immobilized enzymes in food processing* (pp. 135–140). Boca Raton, FL: CRC Press.

Danisman, T., Tan, S., Kacar, Y., & Ergene, A. (2004). Covalent immobilization of invertase on microporous pHEMA-GMA membrane. *Food Chemistry, 85*, 461–466.

Emregül, E., Sungur, S., & Akbulut, U. (1995). Investigation on carrier systems for alpha-amylase enzyme immobilisation. *Macromolecular Reports, A32*(Suppl. 3), 301–308.

Emregül, E., Sungur, S., & Akbulut, U. (1996). Effect of chromium salts on invertase immobilisation onto carboxymethylcellulose-gelatine carrier system. *Biomaterials, 17*(14), 1423–1427.

Erginer, R., Toppare, L., Alkan, S., & Bakir, U. (2000). Immobilization of invertase in functionalized copolymer matrices. *Reactive and Functional Polymers, 45*, 227–233.

Esmon, C. P., Esmon, B. E., Schauer, I. E., Taylor, A., & Schekman, R. (1987). Structure, assembly, and secretion of octameric invertase. *Journal of Biological Chemistry, 262*, 4387–4394.

Garnier, F., Youssoufi, H. K., Srivastava, P., & Yassar, A. (1994). Enzyme recognition by polypyrrole functionalized with bioactive peptides. *Journal of the American Chemical Society, 116*, 8813–8814.

Greenfield, P. F., & Laurence, R. L. (1975). *Journal of Food Science, 40*, 906.

Godbole, S. S., Kubal, B. S., & D'Souza, S. F. (1990). Hydrolysis of concentrated sucrose syrups by invertase immobilized on anion exchanger waste cotton thread. *Enzyme and Microbial Technology, 12*, 214–217.

Hartmeimer, W. (1996). *Immobilized biocatalysts*. Berlin: Springer.

Kirk-Othmer (1986). *Encyclopedia of chemical technology*. New York: Wiley.

Kizilyar, N., Akbulut, U., Ozden, M. Y., Toppare, L., & Yagci, Y., (1999). Immobilization of invertase in conducting polypyrrole/polytetrahydrofuran graft polymer matrices. *Synthetic Metals* 104, 45–50.

Kotzelski, J., & Staude, E. (1996). Kinetics of bonded invertase: asymmetric polysulfone membranes. *Journal of Membrane Science, 114*, 201–214.

Lineweaver, H., & Burk, D. (1934). The determination of enzyme dissociation constant. *Journal of the American Chemical Society, 56*, 658–666.

Mansfeld, J., Forster, M., Schellenberger, A., & Dautzengenberg, H. (1991). Immobilization of invertase by encapsulation in polyelectrolyte complexes. *Enzyme Microbiology Technology, 13*(3), 240–244.

Marek, M., Valentino, O., & Kas, J. (1984). Invertase immobilization via its carbohydrate moiety. *Biotechnology and Bioengineering, 26*, 1223.

Mathias, U., & Alexandra, P. (1997). Polyacrylonitrile enzyme ultrafiltration membranes prepared by adsorption, cross-linking, and covalent binding. *Enzyme and Microbial Technology, 20*, 61–68.

Prodanovic, R., Javonovic, S., & Vujcic, Z. (2001). Immobilization of invertase on a new type of macroporous glycidyl methacrylate. *Biotechnology Letters, 23*, 1171–1174.

Reddy, A. V., Maccoll, R., & Maley, F. (1990). Effect of oligosaccharides and chloride on the oligomeric structures of external, internal, and deglycosylated invertase. *Biochemistry, 29*, 2482–2487.

Selampinar, F., Akbulut, U., Ozden, M. Y., & Toppare, L. (1997). Immobilization of invertase in conducting polymer matrices. *Biomaterials, 18*, 1163–1168.

Sidney, P., & Colowick Nathan, O. (1957). *Methods in enzymology* (vol. III). New York: Kaplan Academic Press Inc., pp. 85–86.

Sungur, S., & Akbulut, U. (1994). Immobilisation of β-galactosidase onto gelatine by glutaraldehyde and chromium III acetate. *Journal of Chemical Technology and Biotechnology, 59*(3), 303–306.

Sungur, S., Elçin, M., & Akbulut, U. (1992). Studies on immobilisation of urease in gelatine by crosslinking. *Biomaterials, 13*(11), 795–800.

Tümtürk, H., Arslan, F., Disli, A., & Tufan, Y. (2000). Immobilization of invertase attached to a granular dimer acid-co-alkyl polyamine. *Food Chemistry, 69*, 5–9.

Tien, C. J., & Chiang, B. H. (1999). Immobilization of α-amylase on zirconium dynamic membrane. *Process Biochemistry, 35*, 377–383.

Yılıdrım, O. ¨, Akbulut, U., Arínç, E., & Sungur, S. (1994). Immobilisation of NADH-cytochrome b5 reductase nto gelatine by crosslinking. *Macromolecular Reports A, 31*(Suppl. 1–2), 19–28.

Zaborsky, O. (1973). In Weast (Ed.), *Immobilised enzymes*. R.C. Ohio: CRC Press.