ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 1 (1995) L1–L6

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Effect of heat treatment on enzymes entrapped into polymer gels

Minoru Kumakura

Department of Bioscience, The Nish-Tokyo University, Uenohara, Kitatsuru, Yamanashi 409-01, Japan

Received 27 February 1995; revised 9 May 1995; accepted 7 August 1995

Abstract

Glucoamylase was entrapped into polymer gel matrices by a polymerization technique using an electron beam accelerator, and the effect of heat treatment on the entrapped enzymes was investigated. The activity of the entrapped enzymes as compared to the un-entrapped enzymes was increased when the energy of the electron beam irradiation used to generate the polymer gels was low (300 keV). The activity of the entrapped enzymes varied with irradiation conditions such as electron beam energy, irradiation dose, irradiation temperature, and the property of polymer gel and the temperature of heat treatment after the irradiation.

Keywords: Polymer gels; Glucoamylase; Heat treatment

1. Introduction

It is known that the immobilization of enzymes leads to an increase of their stability while improving the handling properties of enzymes and improving the efficiency with which they can be used in bioconventions [1–3]. Immobilization is important to maintain constant environmental conditions in order to protect the enzyme against changes in pH, temperature, or ionic strength; this is generally reflected in enhanced stability [1]. Immobilization through entrapment has provided the greatest scope for deploying enzymes in different physical forms. Entrapment is a very attractive method of immobilization when the size differential between substrate and enzyme is large, when the enzyme is very susceptible to chemicals or deactivation and when a strong interaction with the surface of material is undesirable or can not be achieved directly [4]. The most successful techniques for enzyme entrapment use hydrogels since, in general, they stabilize the active structure and are easily wetted, allowing free access of substrates. Entrapment in natural polymer gels such as calcium alginate gel is one of the simplest methods of immobilization. However, there are some limitations, such as low stability and high porosity of the gel [1]. These characteristics could lead to leakage of enzymes, thus generally limiting its use to whole cells or cell organelles [5]. The synthetic polymer gels are almost exclusively formed in situ with enzymes distributed evenly throughout the matrix. In the case of immobilization of enzymes by polymerization using chemical catalysts, many workers have undertaken the polymerization below ambient temperature to reduce heat inactivation of enzymes [2]. So far, polymerization of acrylamide monomers using redox catalysts has been applied for entrapment immobilization of enzymes by many workers, though acrylamide is know to be a toxic monomer [1–3,6,7].

The author has developed radiation polymerization techniques at temperatures (below 0°C) in

SSDI 1381-1177(95)00007-0

which the polymers were formed within 1 h at extremely low temperature (−78°C) using γ-rays from Co-60 source [8,9]. This polymerization technique using various non-toxic monomers has been applied to the immobilization of biological substances such as enzymes, antibodies, and microbial cells [10–13]. Immobilization was best achieved at lower temperatures to block damage to the biological substances during polymerization and promote porosity through phase separation without impurity such as chemical catalysts [11]. In this work, the polymerization reaction using electron beam irradiation was applied to the entrapment of glucoamylase into polymer gel matrix, and the effect of heat treatment on the activity was investigated. The polymerization by electron beam irradiation takes place at shorter irradiation times than that by γ-rays irradiation. This is advantageous for the immobilization of enzymes.

2. Experimental

2.1. Materials

Glucoamylase (Rh. delmer, 1×104 units/g) was obtained from Nagase Sangyo. Reagents (monomers) for the entrappment of the enzyme were hydroxyethyl methacrylate (HEMA), hydroxyethyl acrylate (HEA), nonatetradecaethyleneglycol diacrylate (A-14G), obtained from Shin-Nakamura.

2.2. Irradiation

The enzyme solution (0.5%) containing the monomers was prepared with 0.1 M acetic acid buffer solution, pH 4.5. The enzyme solution was put into an irradiation vessel (6 cm diam. × 1 cm) and irradiated in a low energy electron beam accelerator (300 keV, 5 mA; "Curetron", Nissin-High Voltage Co.) or in a high energy electron beam accelerator (1 MeV, 1 mA; "Dynamitron" IEA-3000-25-2, Dynamitron Co.). The irradiation was carried out using belt-conveyer equipment and the speeds of the conveyers in "Curetron" and "Dynamitron" were fixed to get an irradiation dose of 10 kGy. The irradiation was carried out in nitrogen gas atmosphere. Due to the irradiation the monomers polymerized and caused the enzyme molecules to be entrapped. The polymerization using the electron beam was able to perform for shorter irradiation time (30–60 s) than using γ-rays (1 h).

2.3. Heat treatment

After irradiation, the enzyme solution without monomers was treated at various temperatures for a period of 1 h. The enzyme polymer matrix was immersed in the acetate buffer solution and treated under the same condition as the enzyme solution without monomers.

2.4. Measurement of enzyme activity

The activity of the enzyme was assayed by the measurement of the amount of glucose formed during a 1 h reaction of the enzyme at 40°C with a 5% maltose solution as substrate. The determination of the amount of glucose was carried out using a GL-1 glucose analyzer (Mitsubishi-Kasei). The relative activity (%) was expressed as a ratio of the enzyme activity of the entrapped enzyme to the native enzyme.

2.5. Measurement of water content (hydrophilicity)

The polymers obtained via irradiation were cut and immersed in distilled water at room temperature for a period of one week. The water content of the polymer is expressed as the percentage of the weight of the polymer.

3. Results and discussion

3.1. Effect of entrapment on the heat stability of the enzyme

Effect of heat treatment on the activity of the irradiated enzymes was investigated. The relation-

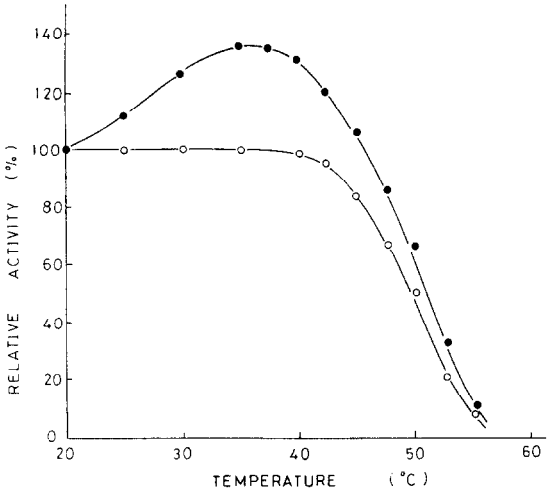

Fig. 1. Relationship between relative activity and treatment temperature of the un- and entrapped enzymes irradiated with a low energy electron beam. Irradiation temperature: 5°C. Monomer: A 14G (concentration; 40%). ○: un-entrapped enzymes; ●: entrapped enzymes.

ship between relative activity and treatment temperature in the un- and entrapped enzymes that were irradiated with the low energy electron beam (300 keV) is shown in Fig. 1. The activity of the un-entrapped enzymes was constant up to 40°C and then decreased due to heat treatment. However, the activity of the entrapped enzymes increased with heat treatment until a temperature of ca. 40°C and decreased at higher temperatures. The maximum activity of entrapped enzymes was at about 35°C. As can be seen in Fig. 1, the relative activity of the entrapped enzymes is larger than that for the un-entrapped enzymes in the temperature range of 20–55°C. Thus, the difference between the activities of the un- and entrapped enzymes appeared by heat treatment. The result shows that the activity of the entrapped enzymes is enhanced via heat treatment. The activity of the un-entrapped enzymes was constant up to 40°C, at higher temperatures it decreased in a similar manner as for the entrapped enzymes. The critical treatment temperature of the entrapped enzymes is high (about 45°C). The decrease of the activity of both un-entrapped and entrapped enzymes at treatment temperature above 45°C is due to heat denaturation of the enzymes.

The relationship between relative activity and treatment temperature in the un- and entrapped enzymes obtained via electron beam irradiation at high energy (1 MeV) is shown in Fig. 2. The irradiation dose during the irradiation of the sample with a high energy electron beam was identical to that with a low energy electron beam. In the case of irradiation with a high energy electron beam, the activity of the un-entrapped enzymes decreased gradually from treatment temperature of about 30°C. The activity of the entrapped enzymes was constant in the temperature range of 20–45°C and decreased at high temperatures. The increase of activity of entrapped enzymes due to heat treatment, that was observed in the irradiation experiment using a low energy electron beam, was not observed, indicating that the increase of the activity is intimately related to the energy of electron beam.

As can be seen in Fig. 1 and Fig. 2, the stable temperature range for the activity in the un-entrapped enzymes becomes smaller when the electron beam energy is increased. This means that the heat stability of the enzymes is decreased due to increasing electron beam energy. In the temperature range (20–45°C) 100% relative activity of the entrapped enzyme generated via a high energy electron beam is observed. The activity is however lower as compared to entrapped enzymes via a low energy electron beam. From the result of Fig. 2, it is found that the activity of the

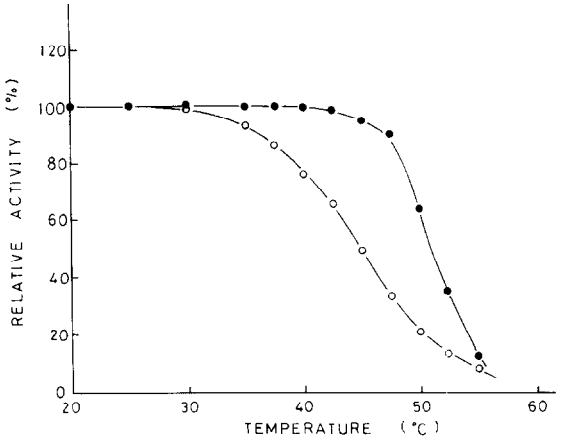

Fig. 2. Relationship between relative activity and treatment temperature of the un- and entrapped enzymes irradiated with a high energy electron beam. Irradiation temperature: 5°C. Monomer: A 14G (concentration; 40%). ○: un-entrapped enzymes; ●: entrapped enzymes.

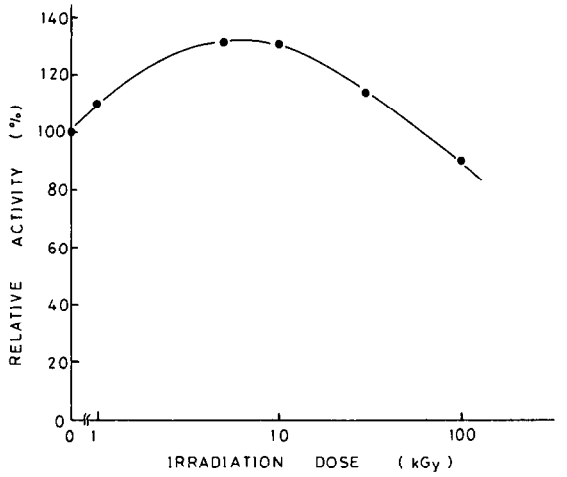

Fig. 3. Relationship between relative activity and irradiation dose for the entrapped enzymes. Irradiation was done with a low energy electron beam. Irradiation temperature: 5°C. Monomer: A 14G (concentration; 40%). Heat treatment: 40°C.

enzymes in the irradiation was markedly protected by entrappment with polymer gel matrix.

3.2. *Effect of irradiation dose on enzyme activity*

The effect of the irradiation dose on the activity of the entrapped enzymes was investigated. The irradiation was done with a low energy electron beam (300 keV) at 5°C and the entrapped enzymes were subsequently treated at 40°C for 1 h. The relationship between relative activity and irradiation dose is shown in Fig. 3. The activity increases upon increasing the irradiation dose, reaches a maximum at ca. 10 kGy, and then decreases. At irradiation doses below 10 kGy, the relative activity of the entrapped enzymes is identical to that of the un-entrapped enzymes. At high irradiation doses, the relative activity decreases due to damage to the enzymes. The irradiation dose giving an increase of the relative activity of the entrapped enzymes was 5–10 kGy. The entrapment of the enzymes is due to a radiation chemical reaction of the monomers such as A 14G which leads to the formation of polymer gel. This radiation polymerization reaction has a threshold energy above which activated species are produced such as radicals finally leading to formation of a polymer gel. This threshold energy in the polymerization reaction of methacrylate and dimethacrylate monomers such as HEMA, A 14G, etc. is at about 5 kGy or above. This threshold energy corresponds with the irradiation dose giving a maximum activity of the entrapped enzymes (Fig. 3). The entrapped enzymes intimately interact with the molecules of the polymer chains which are entangled with each other. Such an entanglement of enzyme molecules with polymer molecules leads to an increased enzyme activity.

3.3. *Effect of temperature during irradiation*

The temperature during irradiation of to be entrapped enzymes was varied in the range of −80°C to 50°C to investigate the effect of the irradiation temperature on the enzyme activity. After irradiation, the enzymes were treated at 40°C for 1 h. The relationship between enzyme activity and irradiation temperature is shown in Fig. 4. The activity of the entrapped enzymes decreases upon increasing the irradiation temperature. At irradiation temperatures below 0°C the activity increased as compared to the activity at an irradiation temperature of 5°C. The monomer reagents (A 14G, HEMA, HEA) for polymerization are polymerizable in the supercooled state via irradiation [8,11]. These monomers are an organic glass-forming substance, which do not crystallize

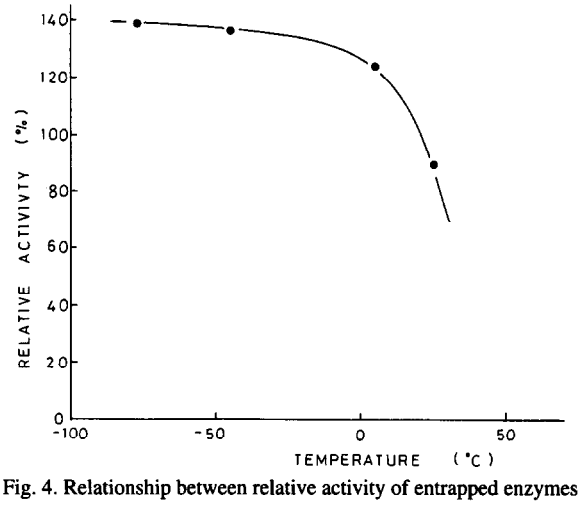

Fig. 4. Relationship between relative activity of entrapped enzymes and irradiation temperature. Irradiation was done with a low energy electron beam. Monomer: A 14G (concentration; 40%). Heat treatment: 40°C.

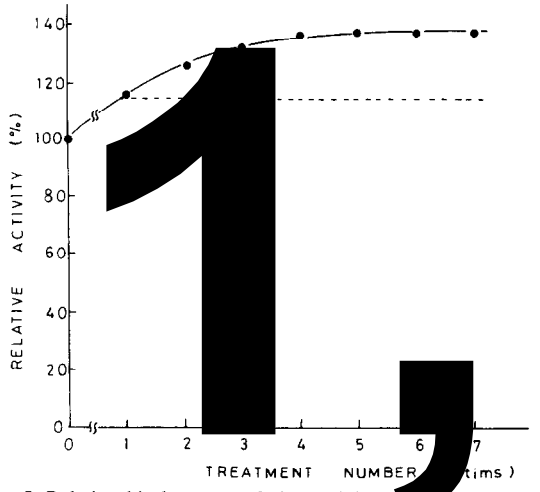

Fig. 5. Relationship between relative activity a… mber of heat treatment cycles for the heating of the un- and entrapped enzymes after irradiation of the enzyme molecule with a low energy electron beam. Irradiation temperature: −78°C. Monomer: A 14G (concentration; 40%). Heat treatment: 40°C. - - -: un-entrapped enzymes; ●: entrapped enzymes.

giving an amorphous glassy state at temperatures below 0°C. This amorphous state was an important factor to keep a stable form of the enzymes at lower temperatures during irradiation [11]. In this work, the enzymes were entrapped into polymer gel resulting from the polymerization of monomer solution. The solution containing monomer, water and the enzymes was transparent corresponding to homogeneous solution at lower temperatures.

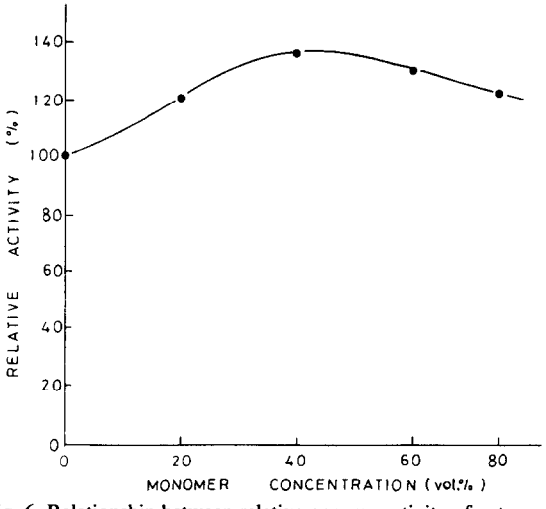

Fig. 6. Relationship between relative enzyme activity of entrapped enzymes and monomer concentration after irradiation with a low energy electron beam. Irradiation temperature: 5°C. Monomer: A 14G (concentration; 40%). Heat treatment: 40°C.

As polymerization proceeds due to irradiation, the enzymes become entangled as the polymers formed from the monomers such as A 14G are hydrophilic reagents. The enzymes become mechanically entrapped in the polymer gel, the structure of the entrapped enzyme molecule could be a deformed state. The state of the entrapped enzyme becomes a more activated one by heat treatment.

The enzyme molecules irradiated at low temperatures are firmly trapped on the surface and/or the interior of the polymer gel. The subsequent heat treatment of the entrapped enzymes was necessary for an appearance of the activity of the enzymes. Heat treatment probably resulted in a rearrangement of the entrapped enzymes which consequently become more active. The increase of the number of heat treatment cycles results in a further increase of enzyme activity (Fig. 5). The relative activity of the un-entrapped enzymes that were irradiated at a temperature of −78°C was slightly increased by heat treatment as shown in Fig. 5. Such increase of activity of un-entrapped enzymes was observed only when irradiation took place at temperatures below 0°C. In the previous work on the irradiation of cellulase, it was observed that the activity of the enzymes that were irradiated at low temperature (−78°C) increased after heat treatment (40°C), whereas the activity of the enzymes irradiated at 4°C decreased after heat treatment. In the case of glucoamylase, the activity of the enzymes irradiated at 5°C did not increase after heat treatment, indicating that the effect of heat treatment on activity of irradiated enzymes is depended on the kind of enzymes.

3.4. *Effect of the property of polymer gel*

The effect of the structure of the polymer gel matrix on the relative activity of the enzyme molecules was investigated. The phase structure of the polymer gel did change as a function of the monomer concentration of the solution before irradiation. In this work, the solution containing monomer, water and enzymes was converted to polymer via irradiation. The physical properties

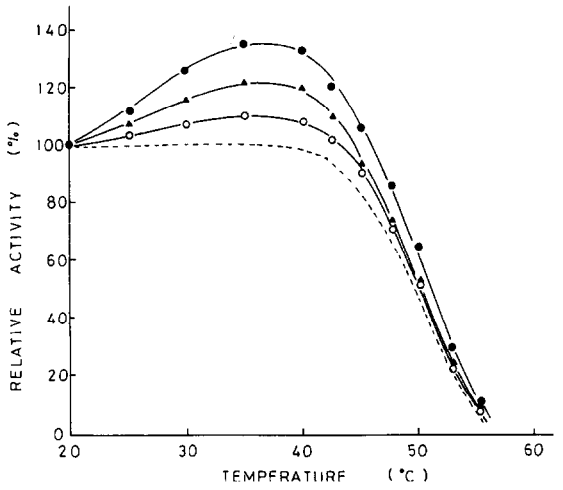

Fig. 7. Relationship between relative enzyme activity of entrapped enzymes and treatment temperature in the un- and entrapped enzymes after irradiation with a low energy electron beam. Irradiation temperature: 5°C. Heat treatment: 40°C. - - -: un-entrapped enzymes. ○: entrapped enzymes using HEA; ▲: entrapped enzymes using HEMA; ●: entrapped enzymes using A 14G.

of the polymer such as phase structure was varied by changing the monomer concentration. The relative activity of the entrapped enzymes did change as a function of monomer concentration as shown in Fig. 6. As monomer concentration increased, the relative activity of the enzymes increases, reaches a maximum and then decreases. The maximal relative activity was obtained with a monomer concentration of about 40%, meaning that the enhancement of the enzyme activity is related to the properties of the polymer. The structure and softness of the polymer are changed as a function of monomer concentration, that is, porous structure and softness are decreased via increasing the monomer concentration.

The decrease of relative enzyme activity at high monomer concentrations is due to a highly rigid entrappment of the enzymes. The results in Fig. 6, suggest that the entrapment of enzymes with a moderate softness can lead to a more suitable state possibly by the control of the mobility of the enzyme and polymer molecules throughout heat treatment. The effect of the softness of the polymer gel onto relative enzyme activity is demonstrated by the experiments using other monomers such as HEMA, HEA as shown in Fig. 7. The water contents of poly-A 14G, poly-HEMA and poly-HEA were 32, 26, and 45%, respectively, corresponding to hydrophilicity or softness of polymer. The relative activity of the entrapped enzymes in A 14G was the highest among the three monomers, suggesting that the polymer gel from A 14G is the most suitable for the activation of the enzyme molecule. A 14G polymer is a cross-linked gel, differing in water content from HEA and HEMA polymer. The enzymes entrapped into a polymer of HEA are surrounded by very soft gels with hydrophilic properties, consequently the relative enzyme activity is similar to that of the unentrapped enzymes.

References

[1] P.S.J. Cheetham, in A. Wiseman (Ed.), Handbook of Enzyme Biotechnology, Ellis Horwood, Chichester, 1985, pp. 74–86.
[2] A. Rosevear, J.F. Kennedy and J.M.S. Cabral (Eds.), Immobilized Enzymes and Cells, Adam Hilger, Bristol, 1987.
[3] W. Hartmeier (Ed.), Immobilized Biobiocatalyst, An Introduction, Springer-Verlag, Berlin, 1986.
[4] A. Klibanov, Science, 219 (1983) 722.
[5] O. Smidsrod and G. Skjak-Braek, Trends Biotechnol., 8 (1990) 71.
[6] R. Sadhukhan, S.K. Roy and S.L. Chakrabarly, Enzyme Microb. Technol., 15 (1993) 801.
[7] T.G. Park and A.S. Hoffman, Enzyme Microb. Technol., 15 (1993) 476.
[8] M. Kumakura, T. Fujimura and I. Kaetsu, Eur. Polym. J., 19 (1983) 621.
[9] M. Kumakura and I. Kaetsu, Polym. Lett., 21 (1983) 609.
[10] M. Kumakura, S. Adachl and I. Kaetsu, Enzyme Microb. Technol., 6 (1984) 23.
[11] M. Yoshida, M. Kumakura and I. Kaetsu, J. Macromol. Sci.-Chem., A14 (1980) 555.
[12] M. Kumakura, I. Kaetsu, M. Suzuki, S. Adachi and K. Imagawa, Anal. Chim. Acta, 161 (1984) 109.
[13] M. Kumakura, I. Kaestu and K. Nisizawa, Biotechnol. Bioeng., 26 (1984) 17.