Biochimica et Biophysica Acta, 315 (1973) 18–21

BBA 66939

PREPARATION OF IMMOBILIZED ENZYMES BY γ-RAY IRRADIATION

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(Received February 12th, 1973)

SUMMARY

Immobilized enzymes were prepared from acrylamide monomer only by means of γ-ray irradiation, a cross-linking reagent such as *N,N'*-methylenebisacrylamide being unnecessary in this method. Glucoamylase, invertase and β-galactosidase were each immobilized by this method. The rigid gel was prepared by using more than 2 M (mega) rad of γ-rays. The immobilized enzymes possessed satisfying activities and no leakage of the enzymes from the gel was detectable.

Many studies have been reported on the preparation of immobilized enzymes[1]. In the polyacrylamide gel entrapping method, the enzyme is physically entrapped in the matrices of the gel network by using acrylamide monomer and *N,N'*-methylenebisacrylamide, a cross-linking reagent, as raw materials for the gel[2]. The present authors have developed a new entrapping method for forming gels from a mixture of enzyme and acrylamide monomer by means of γ-ray irradiation. In this method, a cross-linking reagent, such as *N,N'*-methylenebisacrylamide, is unnecessary and no leakage of the enzyme from the gel is detectable. This new entrapping method using γ-ray irradiation is described below.

Glucoamylase (Diazyme, Miles Laboratories, Elhart, Ind., U.S.A.), invertase (pure grade, Seikagaku Kogyo Co., Tokyo, Japan) and β-galactosidase (Galantase, Tokyo Tanabe Co., Tokyo, Japan) were dissolved in distilled water and the resulting solutions were used after dialysis. Acrylamide monomer was recrystallized from ethyl acetate.

The determination of glucoamylase activity and the definition of the glucoamylase unit have been described in a previous paper[3]. Maltose was used as substrate in this experiment. The determination of invertase activity and the definition of the invertase unit were also described in another paper[4]. Sucrose was used as substrate. Standard estimation of β-galactosidase was as follows: the enzyme reaction was carried out by incubating a mixture of 5 ml of 0.055 M lactose solution, 4 ml of 0.04 M acetate buffer (pH 4.5) and 1 ml of native enzyme solution (or 200–300 mg of the gel) at 40 °C, for 30 min. Incubation was static in the case of the native enzyme and was carried out with shaking at 130 rev./min in the case of the gel. The enzyme re-

action was stopped by heating at 100 °C and the glucose liberated was determined by means of a Glucostat (Worthington Biochemical Corp.). One unit was defined as the activity liberating 1 μmole of glucose, pH 4.5, at 40 °C.

Polyacrylamide gel entrapment by γ-ray irradiation was carried out as follows: 5 g of acrylamide monomer were dissolved in 20 ml of 0.005 M acetate buffer (pH 4.5) saturated with N_2, and 2.5 ml of enzyme solution was added to the solution, with stirring, under a N_2 atmosphere. 5 ml of the enzyme–acrylamide solution was poured into an ampoule. The ampoule was sealed in a N_2 atmosphere and irradiated with γ-rays at a rate of $6.54 \cdot 10^4$ rad/h. Irradiation equipment of Tokyo Sibaura Electric Co., in which ^{60}Co of 500 Ci was placed as the irradiation source, was used. The sealed ampoule was irradiated in close proximity to the irradiation source, with rotation. After irradiation, the gel formed was picked out from the ampoule and was shattered by means of a razor in the case of 1.0 Mrad irradiation and by a homogenizer in the case of more than 2.0 Mrad of γ-rays. The shattered gel was washed 3 times with distilled water and was weighed after draining.

Leakage of enzyme from the gel was assayed as follows. 1 or 2 g of the gel was suspended in a mixture of 20 ml of 0.055 M substrate solution and 20 ml of buffer. In the case of glucoamylase and β-galactosidase, 0.04 M acetate buffer (pH 4.5) was used and in the case of invertase, 0.02 M phosphate buffer (pH 5.2) was used. These suspensions were incubated for 60 min at 40 °C with shaking. After incubation, the suspension was filtered to remove the gel. The filtrate was dialyzed against tap water and the activity remaining assayed.

The effect of γ-ray irradiation on native enzymes was assayed as follows. 1 ml of each enzyme solution was poured into an ampoule and the ampoule sealed in a N_2 atmosphere. The sealed ampoule was irradiated by the procedure described above. After irradiation, enzyme activities of the ampoules were assayed.

Glucoamylase, invertase and β-galactosidase were each immobilized by the new method described above: 108.4 units of glucoamylase (14.4 mg as protein), 2021 units of invertase (3.0 mg as protein) and 130.1 units of β-galactosidase (15.0 mg as protein) were used, respectively. The results are shown in Table I.

The gel prepared using more than 2.0 Mrad of γ-rays was rigid, but that prepared using 1.0 Mrad of γ-rays was quite soft. When the gels were cut by a razor, the major diameter was in the range of 0.3–1.5 mm and the mean major diameter was 0.6–0.7 mm. The form and the surface were irregular. When the gels were homogenized by a blender, the major diameter was in the range of 0.02–0.6 mm and the mean major diameter was 0.05–0.1 mm. The form and the surface were irregular.

In the case of glucoamylase, a complete gel was prepared by irradiation with more than 2 Mrad of γ-rays. When 2 Mrad was applied, 60% of the enzyme activity was lost on entrapping. However, the enzyme was almost completely entrapped and no leakage of it could be detected. These data are similar or rather superior to those for polyacrylamide gel entrapments which have previously been reported.

In the case of invertase, although the invertase was almost completely entrapped by irradiation with 1 Mrad of γ-rays, 75% of the enzyme activity was lost on entrapping. However, leakage was not detectable.

In the case of β-galactosidase, the enzyme was almost completely entrapped by irradiation with 1 Mrad of γ-rays, as with the invertase gel, and 65–70% of the enzyme activity was lost on entrapping. No leakage could be detected.

TABLE I

PREPARATION OF IMMOBILIZED ENZYMES

Expt No.	*Rad*	*Amount of gel (g)*	*Added activity (units)*	*Activity in washings (units)*	*Entrapped activity (A) (units)*	*Total activity of the gel (B) (units)*	*Ratio B/A (%)*	*Leakage of the enzyme*
Preparation of glucoamylase gel								
1	$1.0 \cdot 10^6$	12.0	108.4	8.8	99.6	40.9	41	++
2	$2.0 \cdot 10^6$	9.2	108.4	5.6	102.8	40.8	40	—
3	$4.0 \cdot 10^6$	7.2	108.4	0.0	108.4	16.9	16	—
Preparation of invertase gel								
1	$1.0 \cdot 10^6$	11.2	2021	112	1909	482	25	—
2	$2.0 \cdot 10^6$	8.0	2021	22	1999	442	22	—
3	$4.0 \cdot 10^6$	6.7	2021	6	2015	145	7.2	—
Preparation of β-galactosidase gel								
1	$1.0 \cdot 10^6$	12.2	130.1	4.3	125.8	40.9	33	—
2	$2.0 \cdot 10^6$	9.0	130.1	0.0	130.1	25.8	20	—
3	$4.0 \cdot 10^6$	7.2	130.1	0.0	130.1	0.0	0	—

TABLE II

EFFECT OF γ-RAY IRRADIATION ON ENZYME ACTIVITIES

	Control (units)	*Enzyme activity (units)*		
		$1.0 \cdot 10^6$ rad	*$2.0 \cdot 10^6$ rad*	*$4.0 \cdot 10^6$ rad*
Glucoamylase	329.3	321.4	318.4	269.5
Invertase	7547	6248	5887	4502
β-galactosidase	371.8	316.2	284.5	227.3

As a control, the inactivation of these native enzymes by γ-ray irradiation was investigated in their aqueous solution state and the results are shown in Table II. Consequently, native enzymes were quite stable to γ-ray irradiation.

When the polyacrylamide gel was prepared by this method, the pH in the gel showed a tendency to shift to the alkaline side in proportion to the γ-ray irradiation dose and at the same time, the activities of the gels tended to decrease. This decrease was particularly marked in the case of irradiation with 4 Mrad of γ-rays. The cause of the decrease of the activities is considered not to be due to γ-ray irradiation, but to be mainly due to alkaline shifting.

When an enzyme is entrapped within gel matrices and the gel is used for continuous enzyme reactions, leakage of the enzyme becomes a serious problem. Accordingly, continuous saccharification was carried out using glucoamylase gel and the decrease in the activity (*i.e.* the leakage of the enzyme) was investigated as described below. 2 g of glucoamylase gel prepared by irradiation with 2 Mrad of γ-rays and 5 g of sawdust were packed into a glass column (20 mm $\times$ 300 mm) and 0.028 M maltose solution dissolved in 0.005 M acetate buffer (pH 4.5) was passed through the column at a flow rate of 32 ml/h at 40 °C. The resulting ratio of hydrolysis of maltose solution was 60% and this remained almost constant for 14 days. It was therefore clear that the enzyme did not leak from the gel.

Excellent immobilized enzymes were prepared from acrylamide monomer under γ-ray irradiation as described above. It was previously reported that a gel network could be prepared from polyacrylamide by γ-ray irradiation[5], but application of this technique to the preparation of immobilized enzyme systems has not been reported.

The gel formation from acrylamide monomer is considered as follows: after polymerization of acrylamide monomer, the carbons to which the acid amide is linked are changed to the radicals, by releasing hydrogen by means of γ-ray irradiation, and the radicals are linked together to form the gel matrices. The added enzymes are not considered to be covalently linked to the gel but to be physically entrapped in the gel matrices.

The fact that immobilized enzymes can be prepared by irradiation is considered to be of particular interest. This gel entrapment procedure is so simple that the method is expected to be widely applicable in the field of immobilized enzymes.

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