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Development of Effective Cross-Linking Method for Bioactive Substance—Enzyme Immobilization Using Glutaraldehyde Oligomers

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When a 10% aqueous solution of glutaraldehyde (GA) was alkalized to pH 8.5 in borate buffer solution and heated at 60 °C, the ultraviolet spectrum of GA solution showed two distinct absorption maxima. The one at 280 nm with a weak absorbance ascribable to the C=O bond in the aldehyde group shifted to near 300 nm after 50 min with a slight increase in its intensity. Another maximum at 235 nm with a strong absorbance was ascribable to the C=C bond of the α,β-unsaturated aldehyde group which was formed by aldol condensation reaction of GA monomer, and its absorbance increased markedly with increasing reaction time. The high performance liquid chromatography (HPLC) analysis with detection at 235 nm indicated that several GA oligomers were formed by the alkali treatment and their concentrations increased. The cross-linking ability of these oligomers was examined by immobilizing enzymes (alcohol dehydrogenase (ADH), glutamate dehydrogenase (GLDH)) to an aminated polymer gel matrix by reaction with the treated GA solution. The enzyme activities increased with increasing concentration of GA oligomers. Then, the GA oligomers were isolated and used as the cross-linking agent. The activities of ADH and GLDH were 4-fold and 13-fold higher, respectively, than those obtained by using untreated GA solution, while the total amounts of immobilized enzymes were almost unchanged. These results suggest that GA oligomers may act as cross-linkers in a manner different from the generally accepted Schiff base formation reaction; a possible mechanism may involve addition reaction of an amino group to the double bond in the aldol condensate of GA. This reaction, if it does occur, seems to be effective for the enhancement of the immobilized enzyme activity.

Keywords immobilized enzyme; enzyme activity; alcohol dehydrogenase; glutamate dehydrogenase; glutaraldehyde; glutaraldehyde polymer; cross-linking agent; aminated polymer gel

Glutaraldehyde (GA) has been widely used as a mild cross-linking agent for the immobilization of enzymes, proteins and various bioactive substances bearing amino groups, since the reaction proceeds in aqueous buffer solution under conditions close to the physiological pH, ionic strength and temperature. However, the difficulty lies in obtaining reproducible activity of the immobilized substance; it is sometimes found that the activity of the enzyme immobilized with the use of pure GA solution is rather lower than in the case of GA solution of low grade. This observation suggests that certain impurities in the GA solution may be involved in the immobilization reaction in addition to the expected Schiff base formation reaction. The present paper deals with the improvement of the immobilization method by using GA oligomers as a cross-linking agent.

Experimental

Materials Aqueous 70% GA solution available for use in the electronmicroscopy was obtained from Wako Pure Chemicals, Ltd. (Osaka, Japan). Alcohol dehydrogenase (ADH), glutamate dehydrogenase (GLDH) and β-nicotinamide adenine dinucleotide (β-NAD) were the products of Oriental Yeast Co. (Tokyo, Japan). The aminated polymer gel used for immobilization of enzyme was a gift from Toso Co. (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemicals, Ltd. and Nakarai Tesque, Inc. (Kyoto, Japan). All reagents were used as supplied.

Alkaline Treatment of GA and Activation of Polymer Gels The commercial GA solution was diluted to the final concentration of 10% (pH 8.5) with distilled water and a 0.3 M borate buffer solution. Then the solution was heated at 60 °C in a water bath. Every 10 min after initiation of heating, a portion of the solution was withdrawn and mixed with an equal volume of 0.5 M phosphate buffer solution (final pH 6.5). A 3-ml portion of the mixed solution was added with 0.5 g of aminated gel and incubated at 40 °C for 2 h under gentle shaking. Then the gel was filtered off and washed with 250 ml of water.

Preparation of GA Oligomers The commercial GA solution was diluted to 2% with distilled water, alkalized to pH 8.0 by addition of $NaHCO_3$ (final concentration 0.3%) and 1 M NaOH, and stored at 40 °C for one week. This solution was neutralized with 1 M HCl and fed at a flow rate of 8.5 ml/min into a glass column (3 cm i.d × 16 cm) packed with octylsilyl silica (Sepralyte, 40 μm, 60 Å, carbon content 10%, Analytichem International, California, U.S.A.) which had been washed with 500 ml of acetonitrile then with 500 ml of water. Then a 400 ml portion of water was passed through the column to remove GA monomer, and GA oligomers absorbed were eluted with 200 ml of acetonitrile at a flow rate of 10 ml/min. The eluate was collected, and evaporated almost to dryness under reduced pressure at room temperature. The residue was lyophilized. The high performance liquid chromatography (HPLC) analysis of the residue (Fig. 3) revealed several peaks due to GA oligomers, but no peak of GA monomer. The mixture of GA oligomers thus isolated (white solid) was dissolved in 0.5 M phosphate buffer to make a 4% (w/v) saturated solution. The activation of the polymer gel was achieved in the same manner as described above.

Immobilization of Enzyme An enzyme (ADH or GLDH; 7 mg) and $NaCNBH_3$ (20 mg) were dissolved in 3 ml of 50 mM phosphate buffer solution (pH 7.4). The activated gel (0.5 g) was added and incubated at room temperature for 2 h with gentle shaking. The amount of enzyme immobilized was estimated by Lowry's method[1] from the concentration of enzyme remaining in the incubation solution. The gels were washed with 250 ml of water by filtration with suction.

Assay of Immobilized ADH Activity Immobilized ADH gel (0.05 g) suspended in 22 ml of 10.9 mM pyrophosphate buffer (pH 8.8) containing 1.13 M ethanol, to which 2 ml of 48 mM β-NAD was added under stirring. A 1 ml portion of the suspension was withdrawn at an appropriate time interval and passed rapidly through a membrane filter. The concentration of reduced nicotinamide adenine dinucleotide (NADH) in the filtrate was determined from the absorbance at 340 nm. The immobilized ADH activity was determined from the initial slope of the absorbance *vs.* time plots.

Assay of Immobilized GLDH Activity A 0.13 mg portion of the immobilized GLDH gel was suspended in 22 ml of 0.1 M pyrophosphate buffer solution (pH 9.0) containing 0.1 M monosodium glutamate, to which 2 ml of 60 mM β-NAD was added under stirring. The subsequent procedures were the same as in the case of immobilized ADH mentioned above.

Reversed-Phase Liquid Chromatography HPLC analyses of untreated

and alkali-treated GA solutions were carried out with a model LC-6A liquid chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a model MCPD-3500 multichannel photodiode array ultraviolet (UV) detector (Otsuka Electronics, Japan). A 3 μl portion of sample solution was injected into a column (250 × 4.6 mm i.d.) packed with Inertsil ODS (5 μm, Gasukuro Kogyo Ltd., Tokyo, Japan). Elution was carried out at 55 °C in a gradient mode with solvent A (0.02 M phosphate buffer mixed with acetonitrile at a volume ratio of 9 : 1, pH 2.5) and solvent B (0.02 M phosphate buffer mixed with acetonitrile at a volume ratio of 3 : 7, pH 2.5). The ratio of A to B was 95 : 5 (v/v) for the first 5 min, followed by a linear gradient to 10 : 90 (v/v) over the next 16 min, and isocratic elution was continued thereafter. The flow rate was 1.5 ml/min. The absorption of the eluate was monitored by a photodiode array detector in a range between 200 and 350 nm.

Size-Exclusion Chromatography A 3 ml portion of alkali-treated GA solution was vigorously shaken for several minutes with 3 ml of ethyl acetate. After centrifugation at 3000 rpm for 5 min, a 2 ml portion of the organic layer was evaporated *in vacuo* at room temperature. The residue was dissolved in 2 ml of tetrahydrofuran. A 2 μl portion of the solution was subjected to size-exclusion chromatography(SEC) by using a Shodex KF-803 column (300 × 8 mm i.d.). Elution was carried out at 50 °C with the mobile phase of tetrahydrofuran at a flow rate of 0.5 ml/min. The eluate was monitored by measuring the UV-absorption at 235 nm.

Results

UV Absorption Spectra Figure 1 shows UV absorption spectra of GA solutions treated under alkaline conditions for various periods of time. A weak absorption band at λ_{max} 280 nm observed in the initial state (spectrum a) could be assigned to $n \rightarrow \pi^*$ transition of the C=O bond[2] in the saturated aldehyde group. This band shifted to near 300 nm and gradually increased in intensity with increasing reaction time, which could be ascribed to the formation of α,β-unsaturated aldehyde in the reaction solution. Another strong absorption band at 235 nm observed in the spectra b to f could be due to $\pi \rightarrow \pi^*$ transition of the C=C bond,[2] and its intensity increased markedly as the reaction proceeded. These results, in agreement with previous findings, suggested that GA underwent aldol condensation reaction to yield GA polymers.[3-5]

HPLC Separation The reversed-phase contour chromatograms of untreated and alkali-treated GA solutions are shown in Fig. 2, where the treatment was carried out under the same conditions as in Fig. 1. The peak with a retention time of 3 min seen in all chromatograms is clearly due to GA monomer. Other peaks with longer retention times essentially showed UV absorption only at 235 nm, because the extinction coefficient at 235 nm is much larger than that at 280 nm (see Fig. 1). It is clearly indicated that the untreated GA solution did not include apparent impurities (Fig. 2a), and that the number and the amounts of GA oligomers increased with the progress of the reaction (Fig. 2b to d).

Figure 3 depicts the contour chromatogram of the isolated GA oligomers. The long-time (one week) exposure of GA solution to the alkaline condition resulted in a change in the composition of GA oligomers; one of two major peaks with a retention time of 10.1 min (Fig. 2c and d) disappeared, and the other with a retention time of 9.2 min accounted for over 80% of the total area.

In case the reversed-phase HPLC should fail to detect higher polymers of GA, the ethyl acetate extracts of alkali-treated GA solutions were analyzed by size-exclusion chro-

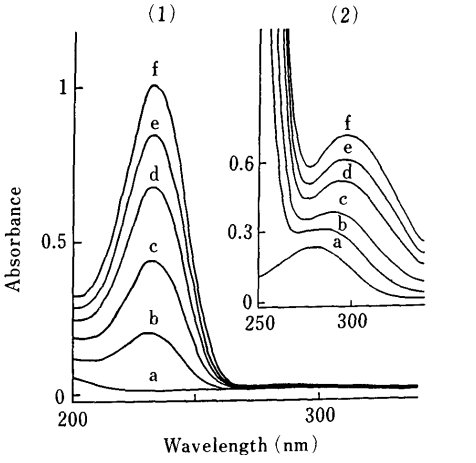


Fig. 1. UV Spectra of 10% GA-Borate Buffer Solution (pH 8.5) Heated at 60 °C for a, 0 min; b, 10 min; c, 20 min; d, 30 min; e, 40 min; f, 50 min
(1) 1000-fold dilution, (2) 20-fold dilution.

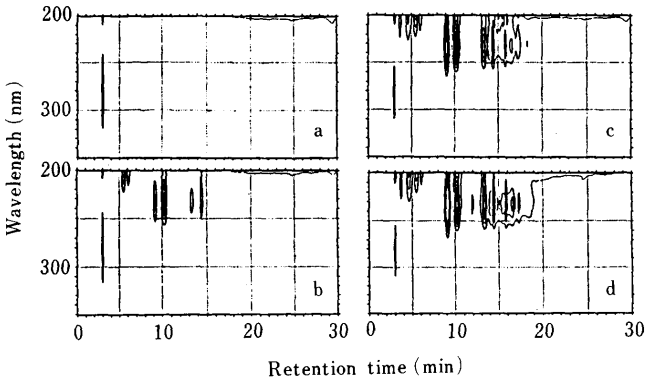


Fig. 2. Reversed-Phase HPLC of 10% GA-Borate Buffer Solution (pH 8.5) Heated at 60 °C for a, 0 min; b, 10 min; c, 30 min; d, 50 min
Separation conditions, see text.

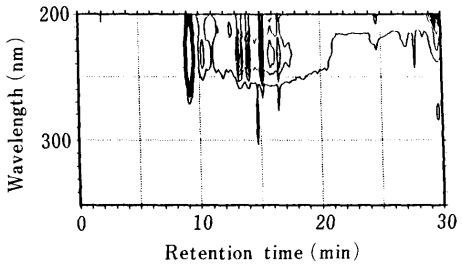


Fig. 3. Reversed-phase HPLC of Isolated GA Oligomers
Separation conditions, see text.

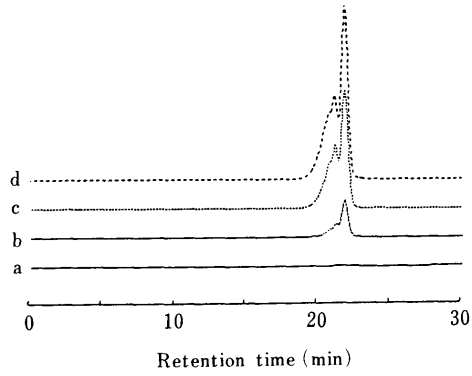


Fig. 4. Size-Exclusion Chromatography of 10% GA-Borate Buffer Solutions Heated at 60 °C for a, 0 min; b, 10 min; c, 30 min; d, 50 min
Separation conditions, see text.

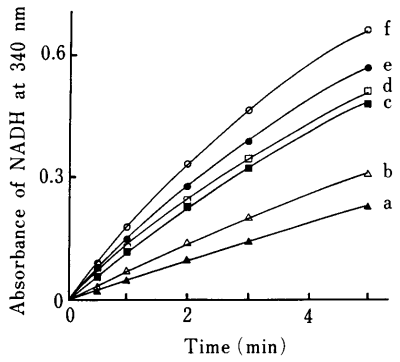


Fig. 5. Time Course of Production of NADH

ADH was immobilized on gel by using GA solution treated in borate buffer (pH 8.5) at 60 °C for a, 0 min; b, 10 min; c, 20 min; d, 30 min; e, 40 min; f, 50 min.

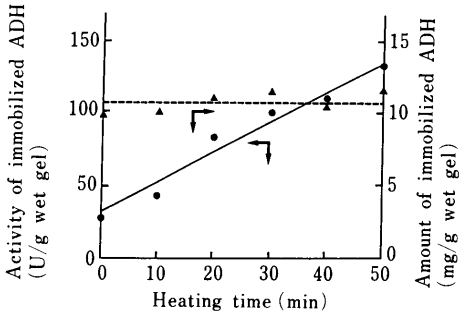


Fig. 6. Activity (——) and Amount (------) of ADH Immobilized on the Gel by Using GA Solution Treated in Borate Buffer (pH 8.5) at 60 °C for 0 min to 50 min

matography. As can be seen in Fig. 4, a longer reaction time resulted in a higher yield of GA polymers, and the molecular weight distribution expanded gradually toward higher degree of polymerization. However, all the peaks were eluted in a relatively narrow range of long retention time, suggesting that high polymers were not formed under the reaction conditions employed.

Activity of Immobilized Enzyme Figure 5 shows the time courses of NADH formed from NAD^+ by oxidation of ethanol with ADH, when ADH was immobilized with the use of GA solution treated under alkaline conditions for various periods of time (Fig. 5a to f). Compared with the result for untreated GA solution (line a), alkali treatment of GA brought about a higher yield of NADH. The enzyme activities were evaluated from the initial slopes of the time course plots. The results are shown in Fig. 6, which also includes the total amounts of enzyme immobilized on the gel. As expected from the results in Fig. 5, the activity of immobilized ADH (shown by a solid line) was increased by the alkali treatment of GA solution, reaching about 4-fold higher by 50 min-treatment, while the amount of immobilized ADH (shown by a dotted line) was almost unchanged. These results suggested that GA oligomers play an important role in the maintenance of immobilized enzyme activity. Then, we isolated the GA oligomers and examined their effects. Table I compares the activities and amounts of ADH and GLDH immobilized on the gels by using untreated GA, alkali-treated GA and isolated GA oligomers as the closs-linkers under the same reaction conditions. It is interesting to note that both enzymes exhibited the highest activities when immobilized with the isolated GA oligomers. The activity ratio was 4 times and 13 times as compared with immobilized ADH and GLDH, respectively, which were cross-linked by the conventional method using untreated GA monomer. The comparison with the results given in Fig. 6 shows that the immobilization of ADH by using alkali-treated GA solution gave a reproducible value of activity, whereas the activity obtained from the untreated GA solution was higher in Table I than in Fig. 6, resulting in a lower value of activity ratio. This means that the use of alkali-treated GA solution is advantageous over the conventional method in obtaining reproducible and high activity of immobilized enzymes.

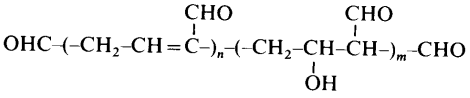
TABLE I. Activities and Amounts of Enzymes Immobilized on the Gel Using Different Cross-Linking Agents

Cross-linker	ADH			GLDH		
	Activity of enzyme immobilized (U/g wet gel)	Activity ratio	Amount of enzyme immobilized (mg/g wet gel)	Activity of enzyme immobilized (U/g wet gel)	Activity ratio	Amount of enzyme immobilized (mg/g wet gel)
GA soln.[a]	73.9	1	9.66	1.79	1	10.7
GA soln. treated[b]	157	2.1	10.7	10.4	5.8	11.0
GA oligomer soln.[c]	295	4.0	11.8	22.5	13	11.1

a) 4% (w/v) GA (untreated). *b*) 4% (w/v) GA heated at 60 °C in borate buffer (pH 8.5) for 50 min. *c*) 4% (w/v) isolated GA oligomer.

Discussion

GA polymerizes by aldol-condensation in aqueous alkaline solution at elevated temperature,[3,4] followed by loss of one water molecule to yield an α,β-unsaturated aldehyde, which exhibits UV absorption with a maximum at 235 nm (extinction coefficient $1.53 \times 10^3\ M^{-1}\ cm^{-1}$)[2] due to π–π^* transition of the C=C bond.[2] The $n \rightarrow \pi^*$ transition of the C=O bond in GA is known to have an absorption maximum at 280 nm (extinction coefficient 3.8—4.4 $M^{-1}\ cm^{-1}$),[2,6] and this λ_{max} value undergoes a red-shift to around 300 nm when the C=O group is conjugated with a C=C bond.[5] This is why the reaction products obtained in the present study were assigned as GA oligomers bearing an α,β-unsaturated aldehyde group in their molecules. One possible chemical structure for this type of GA polymers has been proposed[2] as follows:



The molecular weight distribution of the GA oligomers, as found in Figs. 2 to 4, was expected to range from dimer to pentamer (the minor peaks with retention times between 3.5 and 6.5 min in Fig. 2b, c and d exhibiting UV absorption only at <205 nm are unknown). The major component in the isolated GA oligomers (peak with retention time 9.2 min in Fig. 3) is presumed to be the dimer from preliminary GC-MS analyses. The fact that the three

different cross-linking methods gave different activities but almost the same amounts of immobilized enzymes (Table I), suggests that GA oligomers may bind to an amino group[7,8] by a certain mechanism other than Schiff base formation reaction, and this mechanism may result in high activity of the immobilized enzyme. Although the exact chemical structures of GA oligomers are now being investigated, a possible mechanism may involve Michael-type addition reaction of an amino group and/or a thiol group of the enzyme to the double bond of aldol condensates.[9] The difference in the composition of GA oligomers between Fig. 2 and Fig. 3 may be related in part to the higher activity of the enzymes cross-linked with the isolated GA oligomers than with alkali-treated GA solutions. However, the preparation and isolation of GA oligomers is somewhat tedious, so it would be more practical to use alkali-treated GA solution as a cross-linker. The present study appears to offer the basis for a new and effective cross-linking method to immobilize bioactive materials.

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