Change in Catalytic Property of Trypsin Immobilized on Support Activated with Glutaraldehyde in Lower Protogenic Solvent

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A variety of glutaraldehyde (GA) activated-aminopropyl controlled pore glass supports were prepared in lower protogenic solvents (methanol, ethanol, and *n*-propanol). When trypsin was used as the ligand, the amount of trypsin immobilized on them decreased in the order of water, methanol, ethanol, and *n*-propanol; the maximal reduction of 22% was observed in *n*-propanol versus that in water. The Michaelis constants of the trypsins immobilized on the support activated with GA in alcohols, especially ethanol and propanol, were smaller than that in water, and the minimum K_m value of 1.84×10^{-1} mM was obtained in propanol (4.29×10^{-1} mM in water). During the reaction of GA with 2-aminoethanol, the amount of the aldehyde group from GA being capable of reacting with 2-aminoethanol significantly decreased when the reaction was done in *n*-propanol (0.18 mmol/assay volume, cf. in water; 0.35 mmol/assay volume). The gel permeation chromatography (GPC) measurement of the GA complex, glycine-GA-phenethylamine, also revealed the suppression of the GA self-polymerization in the lower protogenic alcohol as a support activating solvent.

Keywords: glutaraldehyde, enzyme immobilization, solvent effect

Glutaraldehyde (GA), a bifunctional cross-linking reagent, has been extensively used for immobilizing enzymes on a support due to its high reactivity under mild conditions (Peters & Richards, 1977). Although it is being frequently used, the prepared immobilized enzyme has some disadvantages such as the lack of long-term stability (Lappi *et al.*, 1976), poor reproducibility of activity (Nakagawa *et al.*, 1989), and so on.

The reactivity of free GA in aqueous or buffered solution has already been clarified in detail; GA polymerizes through hydration and the aldol condensation reaction under acidic and alkaline pH conditions, respectively (Korn *et al.*, 1972; Holmquist & Lewin, 1991; Ukeda *et al.*, 1991). It has been demonstrated that in both polymerization reactions, the proton addition occurs during the formation steps of the cyclic carbonium ion and the aldol under acidic and alkaline pH conditions, respectively (Hardy *et al.*, 1972). This leads to the suggestion that the self-polymerization reactivity of GA might be suppressed in lower and/or nonprotogenic solvents.

In our previous paper, we prepared the GA spacer complex, glycine-GA-phenethylamine (GGP), *in situ* by using the support introduced *N*^α-9-fluorenylmethyl-oxycarbonylglycine (Fmoc-Gly-O-polymer) in order to analyze the structure of the GA spacer formed on the support (Matsui *et al.*, 1997). The ¹H-NMR spectroscopic and GPC measurements of GGP prepared with 5% GA at pH 10 showed that the GA spacer involved in the enzyme immobilization would be a heteropolymer.

In this study, we prepared the GA activated support without a heterogenous spacer structure using lower proto-

genic solvents, alcohols, as the support-activating solvent. The effect of the protogenicity on the catalytic property of immobilized trypsin was also investigated in this study.

Materials and Methods

Materials GA solution (70%) was purchased from Wako Pure Chemical Industries, Co. (Osaka). Aminopropyl controlled pore glass (aminopropyl CPG, average pore size: 700 Å, 80–120 mesh, amino group content 100 μmol/g-dry gel) and bovine pancreas trypsin (9000 U/mg solid) were obtained from the Sigma Chemical Co. (St. Louis, MO). Fmoc-Gly-O-polymer (glycine content; 0.7 mmol/g-support) was the product of Kokusan Chemical Works, Co. (Tokyo). Trifluoroacetic acid-*d* with the isotopic purity of 99.9% atom D was purchased from Aldrich (Milwaukee, WI). Other reagents were of analytical grade and used without further purification. The deionized water prepared by the Milli-Q Labo (Nihon Millipore Ltd., Yonezawa) was used throughout this experiment.

Determination of the amount of reactive groups produced on the support The amount of reactive groups introduced by the GA activation of the aminopropyl CPG support was estimated from the binding amount of glycine used as a model of the ligand. First, aminopropyl CPG (100 mg) was activated in 2 ml of 5% GA solution at 20°C for 2 h. The 5% GA solution was individually prepared by diluting 70% GA with the following support activating solvents: water, methanol, ethanol, and *n*-propanol. The GA activated aminopropyl CPG support was thoroughly rinsed with water to remove any excess amount of GA. The support was then subjected to a blocking treatment with 2 ml of 0.1 M glycine solution in 0.2 M carbonate buffer (pH 10) at 20°C for 2 h.

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After the blocking, the amount of unreacted free glycine in the supernatant was determined by the 2,4,6-trinitrobenzene sulfonate (TNBS) method (Hazra *et al.*, 1984) to estimate the amount of reactive groups introduced by the GA activation of the aminopropyl CPG support. The support, reduced with 2 ml of 5% NaBH₄ solution after support activation, was used as the control and subjected to the same blocking treatment described above to take into consideration the estimating of the amount of nonspecific absorption of glycine on the support.

Preparation of immobilized trypsin Two milliliters of trypsin (2 mg/ml) in 50 mM phosphate buffer (pH 8) containing 0.5 M NaCl was added to the GA activated aminopropyl CPG support (100 mg) and incubated at 20°C for 2 h. After being thoroughly rinsed with water, 2 ml of 0.1 M glycine solution was added to block the remaining reactive groups at 20°C for 2 h, and then reduced with 2 ml of 0.5% NaBH₄ solution at 20°C for 1 h. The preparations were kept at 4°C in Carmody buffer (pH 3; 100 mM boric acid/25 mM citric acid/50 mM trisodium phosphate) unless otherwise specified.

In the experiment to determine the long-term stability of the preparations, they were stored in Carmody buffer (pH 8) at 4°C until the assay of their residual activity was done.

Determination of the amount of immobilized trypsin Similar to the procedure for determining the reactive groups on the support described above, the amount of immobilized trypsin was indirectly determined from the difference in the absorbance at 280 nm of the supernatant before and after immobilization.

Assay for immobilized trypsin activity The trypsin immobilized on the aminopropyl CPG support (10 mg) was suspended in 2 ml of Carmody buffer (pH 7-12) solutions containing 0.8 mM *N*^α-benzoyl-L-arginine ethyl ester (BAEE) as a substrate, and incubated at 25°C for 2 min with stirring to measure the initial velocity. During the measurement of the kinetic constants, the BAEE concentration was set in the range of 0.1 mM to 0.8 mM. The concentration of hydrolyzed BAEE was determined by the method of Schwert and Takenaka (1955) to estimate the immobilized trypsin activity. One unit of trypsin per mg was defined as the amount of enzyme to hydrolyze 1 μmol of BAEE per min under the above assay conditions.

Preparation of Glycine-GA-Phenethylamine The preparation of the GA spacer complex, GGP, was performed using the procedure as described in our previous paper (Matsui *et al.*, 1997). First, 50 mg of the Fmoc-Gly-*O*-polymer support was incubated with 2 ml of 20% piperidine/*N,N*-dimethylformamide (DMF) at ambient temperature for 15 min to remove the Fmoc groups. After being rinsed thoroughly with DMF and water, the support was subjected to the GA activation treatment. That is, 2 ml of 5% GA solution was added to the support and incubated at 20°C for 2 h. In this experiment, the GA solution was prepared by diluting 70% GA with water, methanol, ethanol, or *n*-propanol. After the incubation, the GA activated support was washed with water, followed by the addition of 2 ml of 0.5 M phenethylamine solution to block any remaining reactive groups. After the blocking reaction at 20°C for 2 h, the reduction was carried out by adding 2 ml of 5% NaBH₄

solution in 0.2 M carbonate buffer (pH 10) at ambient temperature for 1 h to prevent the dissociation of the Schiff base formed between the amino group from phenethylamine and the aldehyde group from GA. After the reduction, the formed GA complex, GGP, was successfully cleaved from the support by 2 ml of 50% CH₂Cl₂/TFA at ambient temperature for 1 h. Finally, the cleaved GGP was obtained by the precipitation using diethyl ether and then evaporated to dryness.

Evaluation of the reactivity of free GA with 2-aminoethanol The reactivity of free GA with 2-aminoethanol in various solvents was evaluated from the amounts of the reacted amino and aldehyde groups. Namely, 0.05 mmol of 2-aminoethanol was added to 0.5 ml of 5% GA solution prepared by dilution of 70% GA with water or the alcohols, and the solution was incubated at 20°C. After incubation, 0.5 ml of 0.25 M NaBH₄ solution in 50 mM NaOH was added to the solution. The solution was diluted by 5 ml of 0.1 M HCl, and then the amount of the amino groups in the solution was determined by the TNBS method (Hazra *et al.*, 1984). Briefly, an aliquot (25 μl) of the sample solution was added to 500 μl of a borate buffer (pH 9.1), followed by the addition of 25 μl of 0.1 M TNBS in a 0.1 M Na₂HPO₄ solution, and incubated at 37°C for 20 min. After adding 4.5 ml of Na₂SO₄ in a 0.2 M NaH₂PO₄ solution, the absorbance at 416 nm was measured. The determination of the aldehyde groups was carried out by the 2,4-dinitrophenylhydrazine (DNP) methods (Jordan, 1967). Namely, an aliquot (5 μl) of the sample solution before being reduced was added to 1.0 ml of the DNP saturated solution in methanol, and then the mixture was incubated at 50°C for 30 min. After adding 5.0 ml of 10% potassium hydroxide in methanol, the solution was allowed to stand at ambient temperature for 30 min, and then the absorbance at 400 nm was measured.

GPC measurement of GA Complex The apparent molecular weight distribution of the GGP was estimated by GPC measurement on an Asahipak GS-220 column (7.5 mmφ×500 mm, Asahikasei Co., Tokyo) using high pressure liquid chromatography (HPLC; LC-9A, Shimadzu Co., Kyoto). The elution was done with 20% CH₃CN in 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min. The monitoring absorbance was set at 260 nm from the maximum absorbance wavelength of phenethylamine as a ligand. The molecular weight distribution of GGP was estimated using angiotensin I (human, M.W. 1296), Tyr-Ile-His-Pro-Phe (M.W. 675), and Gly-L-Tyr (M.W. 238).

NMR measurement of GA Complex The GGP redissolved in trifluoroacetic acid-*d* was placed in a 5-mmφ NMR sample tube and then subjected to ¹H-NMR analysis. The NMR spectrum was obtained using an A400 NMR instrument (400 MHz, JEOL Co., Tokyo) with tetramethylsilane as the internal standard. The operating conditions were as follows: 128 scans were accumulated with spinning at 12 Hz, of a 3.69 μs pulse width (35°), and a pulsed delay of 10.0 s.

Results and Discussion

In order to evaluate the efficacy or usefulness of alcohols as a support activating solvent, the binding amount of glycine for the aminopropyl CPG supports activated with GA in water, methanol, ethanol, and *n*-propanol was mainly inves-

tigated. As shown in Fig. 1, the maximum binding amount of glycine was obtained in water (582 $\mu\text{mol/g-dry gel}$). Assuming that the amino group on the support stoichiometrically reacts with one aldehyde group of the GA monomer to form a Schiff base, the binding amount of the glycine to the GA carbonyl group should be the same as that of the amino groups on the support (100 $\mu\text{mol/g-dry gel}$). However, the binding amount of glycine obtained in water was ca. 6-fold higher than that of amino groups on the support. This indicates that the GA after support activation treatment would have more binding sites to glycine, resulting from any polymerized GA structures with an α,β -unsaturated bond (Walt & Agayn, 1994) as well as the carbonyl group. In alcohols, the binding amount of glycine decreased as compared in water. The minimum binding amount (439 $\mu\text{mol/g-dry gel}$) was obtained when *n*-propanol was used as a support activating solvent. This result would make it possible to explain that the proton addition may be essential for the first step of the self-polymerization of GA. Based on Dimroth and Reichardt's E_T value (Dimroth *et al.*, 1963) as an index of molecular polarity, the decreasing behavior of the binding

amount of glycine shown in Fig. 1 was in accordance with the decrease in the E_T value or ionization ability of the solvent (water, 15.1; methanol, 13.3; ethanol, 12.4; *n*-propanol, 12.1 kJ/mol). When acetonitrile, which is an aprotic solvent, was used for support-activation, the binding amount of glycine increased to 130%, as compared in water, though the data are not shown. Thus, a small amount of protons in the support activating solvent would be needed to suppress the GA self-polymerization reaction on the support.

Similar attempts were done using bovine pancreas trypsin. As shown in Fig. 2, the amount of immobilized trypsin decreased when the lower protogenic solvents were used for support activation, as compared with in water, being similar to the results of glycine binding behavior shown in Fig. 1.

A further investigation of the catalytic properties of immobilized trypsin prepared in various support activating solvents was then done. As shown in Fig. 3, the immobilized trypsin on the supports activated with GA in water and methanol showed almost the same pH-activity profile toward the BAEE. Although the optimal pH of each immobilized trypsin shifted to the alkaline region (water and methanol, pH 10; ethanol and *n*-propanol, pH 9), compared to free trypsin (pH 8), their pH-activity profiles were similar to that of free trypsin. The shifts to the alkaline region agreed with the result reported by Glassmeyer and Ogle (1971) using *N* $^\alpha$ -benzoyl-

Fig. 1. Effect of solvent for support-activation with GA on the amount of bound glycine.

Fig. 2. Effect of solvent for support-activation with GA on the amount of immobilized trypsin.

Fig. 3. Effect of solvent for support-activation with GA on the activity of immobilized trypsin. Trypsin (□) and immobilized trypsin on the aminopropyl CPG activated with 5% GA prepared by diluting 70% GA with water (○), methanol (●), ethanol (△), and *n*-propanol (▲).

Table 1. Kinetics of the immobilized tryptins on the aminopropyl CPG activated with GA in various solvents.

Solvent	K_m (mM)	V_{max} ($\mu\text{mol/min/mg}$)
Water	4.29×10^{-1}	1190
Methanol	4.20×10^{-1}	1080
Ethanol	2.31×10^{-1}	1050
<i>n</i> -Propanol	1.84×10^{-1}	950
Free	2.67×10^{-2}	9530

The immobilized trypsin (10 mg) was added to 2 ml of Carmody buffer (pH 8) containing BAEE (0.1 to 0.8 mM), and incubated at 25°C for 2 min.

DL-arginine-*p*-nitrophenylanilide as a substrate. Levin *et al.* (1964) have also reported that the shift in optimum pH between 9–10.5 of trypsin immobilized on polyanionic copolymers was due to the attraction of hydrogen ion from the surrounding aqueous medium to the negatively charged support by the electrostatic force. As a result, the pH value in the microenvironment near trypsin became lower than the pH measured in bulk, so that the optimal pH apparently shifted to the alkaline region. Thus, the carboxyl group from glycine used to block the remaining reactive groups would be attributed to the negative charge at the surface of the support; a less negative charge of the support activated in ethanol and *n*-propanol than the other solvents showed the lower binding amounts of glycine.

In addition, the measurement of kinetics at pH 8 of each immobilized trypsin was done (Table 1). As a result, the immobilization of trypsin brought about an increase in the Michaelis constant, compared with free trypsin in a similar manner to that reported by Widmer *et al.* (1973). However, the Michaelis constants of the trypsin immobilized on the support activated with GA in alcohols, especially in ethanol and *n*-propanol, were smaller than that in water, and the minimum K_m value of 1.84×10^{-1} mM was obtained in *n*-propanol (4.29×10^{-1} mM in water). These results suggested that the trypsin immobilized onto the aminopropyl CPG support activated with GA in the alcohols would be more flexible or have higher affinity toward the substrate than that in water.

The long-term stability during storage at pH 8 and 4°C of each immobilized trypsin was then investigated. As shown in Fig. 4, the activities of all the immobilized tryptins rapidly decreased with their storage period up to 21-days storage and then gradually decreased. The highest relative residual activity after a 28-day storage (87.7%) was obtained when methanol was used for support activation. It was approximately similar to that reported by Dillon and Wade (1976). They showed that trypsin immobilized onto fibrin with GA retained about 90% of the residual activity after 33-days storage at pH 8.

Following this, the reactivity of GA itself or with 2-amino-

ethanol was investigated to confirm the suppression of GA reactivity in the alcohols. The time course of the reacted amino groups from 2-aminoethanol determined by the TNBS method indicated that ca. 90% of the initial amount of 2-aminoethanol rapidly reacted with GA within a 5-min incubation in water, and afterward, no reaction occurred (Fig. 5). Okuda *et al.* (1991) showed that 6-aminohexanoic acid completely reacted with GA in 0.1 M sodium phosphate buffer (pH 7) when the concentration of GA was more than 3-fold higher than that of 6-aminohexanoic acid. In our experiment, 2-aminoethanol did not completely react with GA in water though the concentration of GA was 5-fold higher than that of 2-aminoethanol. This may be caused by the low reactivity and reversible reaction between 2-aminoethanol and GA. Although the data are not shown, the rapid reactivity of 2-aminoethanol with GA in the alcohols, as well as in water as shown in Fig. 5, revealed that the reactivity of 2-aminoethanol with GA was independent of the protogenicity of the matrix. On the other hand, as shown in Fig. 6, the amount of reacted aldehyde groups from GA after a 2-h incubation in the alcohols was lower than that in water (0.35

Fig. 5. Time course of the amino groups of 2-aminoethanol reacted with GA in water.

Fig. 4. Long-term stability of immobilized tryptins in Carmody buffer (pH 8.0) at 4°C. The symbols are identical to those described in Fig. 3.

Fig. 6. Effect of solvent on the amount of the aldehyde group from GA reacted with 2-aminoethanol after a 2-h incubation at 20°C.

Fig. 7. Gel filtration chromatography of glycine-GA-phenethylamine prepared in various solvents for support-activation. The sample solution was applied to an Asahipak GS-220 and eluted with 20% CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min while monitoring absorbance at 260 nm. The molecular weight distribution was estimated using (a) angiotensin I (human, M.W. 1296), (b) Tyr-Ile-His-Pro-Phe (M.W. 675), and (c) Gly-L-Tyr (M.W. 238).

mmol/assay volume), and the minimum amount was obtained when *n*-propanol was used (0.18 mmol/assay volume). The molar ratios of the reacted aldehyde groups to the reacted amino groups were 7.76, 4.42, 4.41, and 3.91 in water, methanol, ethanol, and *n*-propanol, respectively, indicating that the number of aldehyde groups reacting with one amino group decreased in the alcohols compared to that in water. In aqueous solution, it was reported that the ratio of the aldehyde group from GA assayed by the consumption of NADH by alcohol dehydrogenase to the amino group from 6-aminohexanoic acid was ca. 5 in the presence of excess amounts of aldehyde groups (Okuda *et al.*, 1991). Although the direct comparison of the reported ratio with our results was not possible because of the difference in the assay system of the aldehyde groups, the decrease in the molecular ratio with the protogenicity of the alcohols clearly indicated that the use of alcohols may be profitable for the preparation of the homogeneous GA activated support.

Furthermore, to obtain useful information about the GA spacer structure formed in the alcohols *in situ*, the structural analysis of GGP prepared in the alcohols was done. As shown in Fig. 7, the molecular weight range (600–1300) of GGPs prepared separately in alcohol and water was almost the same. However, the distribution pattern was different due to the support-activating solvents, and the higher molecular weight species of GGP decreased with protogenicity of the solvent. Though the data are not shown, the $^1\text{H-NMR}$ measurement of GGP prepared in water showed that one molecule of phenethylamine was assumed to react with polymerized GA formed by at least 2.8 molecules on average on the support according to the ratio of the integrated value of the methylene signals (δ_{H} 1.3–2.5) from polymerized GA to that of the benzene ring signals (δ_{H} 7.1–7.5) from phenethylamine. In the alcohols, condensed GA formed

using ca. 1.7 molecules was thought to react with one molecule of phenethylamine.

In conclusion, the catalytic properties of trypsin immobilized on the support activated with GA in the alcohols were significantly different from that in water. The stoichiometric analysis of the reaction of GA with 2-aminoethanol and the GPC analysis of GA complex prepared in the alcohols indicated that the use of a lower protogenic alcohol as a support activating solvent would be effective for suppressing the GA self-polymerization or increasing the homogeneity of the GA activated support.

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