Analytical Methods for the Detection and Purification of ε-Poly-L-lysine for Studying Biopolymer Synthetases, and Bioelectroanalysis Methods for Its Functional Evaluation

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This article describes new analytical methods for studying biopolymer ε-poly-L-lysine (εPL). The produced amount of εPL in culture broth can be determined based on the precipitation of polycationic εPL with a colored heteropolytungstate anion and the color change of the supernatant. The product can be separated and purified by precipitation with the tetraphenylborate anion and reprecipitation in the form of the hydrochloride salt. These methods have been applied advantageously to the screening of εPL-synthetase. Also, pyrophosphate can be determined colorimetrically based on the formation of 18-molybdopropophosphate species. The pyrophosphate determination has been successfully applied to the assay of adenylation enzyme, which plays important roles in the biosynthetic mechanism. Under certain conditions, εPL associates with a redox enzyme, glucose oxidase. The effect of the adduction on the stability and reaction rate of the enzyme can be evaluated by measuring the bioelectrocatalytic current, which is related to the enzyme activity. Electrochemical studies showed new applications of εPL as an enzyme stabilizer and a reaction enhancer.

Keywords ε-Poly-L-lysine, colorimetry, separation, enzyme assay, bioelectrocatalytic current

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1 Introduction

ε-Poly-L-lysine (εPL, Fig. 1) is a homopolymer liked by the peptide bond between carboxyl and ε-amino groups.1,2 Industrially, εPL has been produced by a fermentation process using Streptomyces albulus. To date, εPL consisting mainly of 25 – 35 L-lysine residues has been produced and used as a

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natural preservative in food products. Recently, a synthetic enzyme, εPL-synthetase, was identified, and the biosynthetic mechanism has been elucidated. Thus, screening the synthetic enzyme to produce higher- or lower-molecular-weight εPL species as well as a more efficient one has become an important subject. Also, the bioengineering study has been extended to a biologically active substance having lysine oligopeptides.

Thus, we have developed analytical methods useful in bioengineering studies for εPL. Under the acidic and neutral conditions, εPL exists in a polycationic form by protonation of the α-amino group. We found that a colored heteropolymolybdate anion, 12-molybosilicate ([SiM06(OH)6]6–), can be used as a precipitation reagent for the polycationic εPL. On the basis of our finding, a colorimetric εPL microassay has been developed. We also developed a method for the separation of εPL from a culture broth, which is based on precipitation of the polycationic εPL species with tetraphenylborate (TPB–) anion, and reprecipitation in the form of the hydrochloride salt. Compared with the conventional column separation, the method is rapid and easy to carry out. Furthermore, we developed a colorimetric method for the determination of pyrophosphate (PPi) anion. The PPi determination has been applied to the assay of adenylation enzyme, which plays an important role in the biosynthetic mechanism. These methods have been successfully applied to studying not only εPL, but also streptomycins containing β-lysine oligopeptides. This article describes these analytical methods.

Recently, it has been shown that some cationic polymers can be used as an enzyme stabilizer in solution. The stabilizing effect can be ascribed to electrostatic association of the cationic polymers with the negative charges of the enzyme surface to form a polyanion complex species stable in solution. The effect can be studied by a bioelectrocatalysis method in which the inactivation process is followed by the enzymatic bioelectrocatalytic current. Unlike the conventional spectrophotometric enzyme assay, this method can measure the enzyme activity in solution continually and directly, so that the rate constants and kinetic parameters for the thermal inactivation of enzymes can be determined advantageously. Using the bioelectrocatalysis method, εPL has been found to be a superior stabilizer for glucose oxidase (GOD). Furthermore, it has been shown that the GOD-catalyzed oxidation of glucose with a negatively charged oxidant is enhanced remarkably in the presence of εPL. This article also describes the application of the bioelectrocatalysis method for the evaluation of εPL as an additive for enzymatic reaction media.

2 Analytical Methods for the Bioengineering Study of εPL

The acid-base equilibrium or pH dependence of the charge number is an important character of εPL. Although εPL is a polycationic base, the pH titration curve clearly gives an equivalence point, as shown in Fig. 2. The equivalence point pH was around 4, so that εPL exists in the fully protonated form (εPLH4+; n being the degree of polymerization) at pH < 4. Also, the pH at the half equivalence point was determined to be 7.5, and the value is taken to be apparent pK of εPLHn+ (abbreviated as pK in this article).

2-1 Colorimetric εPL assay for the screening of the synthetic enzyme

It has been known that cationic polymers associate with amphiphilic or hydrophobic anions to form sediments. Thus,
εPL has been traditionally assayed on the basis of color fading of the supernatant of the εPL-anionic dye mixture. However, since the precipitation reaction does not occur quantitatively, εPL could not be detected sensitively with anionic dyes, such as methyl orange and picrate. It has been well known that cationic polymers, such as poly(diallyldimethylammonium chloride) and glycolchitosan, can be determined by colloidal titration with an anionic polymer. However, the polycationic εPL did not form the colloidal particle with the anionic polymers, such as poly(vinyl sulfate). We found that a multivalent heteropolyheterometallate anion [SiMo12O40]4– can be used as a superior precipitation reagent for the polycationic εPL. The procedure for the εPL microassay based on the precipitation is illustrated in Fig. 3.

A 40-μL aliquot of sample solutions is transferred into wells in a microtiter plate. For deproteination, 8 μL of the 3 M CCl3COOH aqueous solution is added into each well, and the mixture is centrifuged. The mixture is buffered at pH 2.5 by the addition of 48 μL of the 2 M CH3COOH and 1.5 M NaOH aqueous solution. A Si(IV)–Mo(VI) solution, containing 5% glucose, 1% (NH4)2SO4, 0.5% yeast extract, and 0.004% FeSO4·7H2O, and 0.003% ZnSO4·7H2O, and 0.008% Na2MoO4, 0.002% MgSO4·7H2O, and 0.003% NaH2PO4, 0.002% KH2PO4, 0.001% Na2SiO3, and 0.001% MgSO4·7H2O (pH 6.8) is added into the tube. It is noted that the TPB– anion is not overlapped with those of the polar culture components. Thus, a sample pretreatment is required for HPLC analysis.

We found that εPL can be separated based on the precipitation reaction with the tetraphenylborate TPB– anion.9 The procedure is illustrated in Fig. 5. A 100 μL aliquot of a sample solution is transferred into a microtube, and 10 μL of a buffer solution (pH 3.5) containing 1 M CH3COOH and 50 mM NaOH is added into the tube. It is noted that the TPB– anion is not decomposed immediately in the weakly acidic solution. By adding 100 μL of the 0.2 M NaTPB solution, εPLHn+ forms a precipitate of 1:n stoichiometry, εPL(TPB)n.

As described above, the culture medium for the production of εPL contains K+ and NH4+ ions. After cultivation, various amine compounds would also be produced in the culture broth. Since the TPB– anion forms precipitates with K+, NH4+, and some amine compounds, a mixed precipitate would be obtained from the culture broth. However, the polyelectrolyte salt εPL(TPB)n is insoluble in organic solvents, such as acetone (AC) and 1-butanol. On the other hand, the monovalent cation-TPB– salts are soluble in polar organic solvents. Thus, εPL(TPB)n can be separated from the mixed precipitate by washing with AC. The εPL(TPB)n can be purified further by washing with 1:1 (v/v) AC-water (W) to remove hydrophobic impurities. It has been known that, under strong acidic condition, the TPB– anion is decomposed immediately into benzene and triphenylborane. Therefore, the εPL(TPB)n precipitate is mixed with 10 μL of 1 M HCl. In the reaction mixture, the hydrochloride salt εPL(HCl)n would be formed according to the following reaction: εPL(TPB)n + nHCl → εPLHn+ + nCH3COOH + nNH4+:εPLHn+. The addition of larger volume AC, εPLHCln is precipitated from the mixture. Simultaneously, CH3COOH and n(CH3)4N dissolve into the solvent.
After removal of the supernatant, the εPLHCln precipitate is purified by washing with 20:1 (v/v) AC–W.

As mentioned above, the εPLHn+ species can be precipitated with the [SiMo12O40]4– anion. Also, it has been found that dicarbollylcobaltate forms a precipitate with εPLHn+. These anions do not precipitate with the NH4+ and K+ ions. However, it is not easy to obtain εPLHCln from εPLHn+ salts with the anions.

Figure 6a shows the chromatogram of the εPL-producing culture broth after deproteinization with CH3Cl. It is confirmed that εPL species with \( n = 25 \text{--} 35 \) were produced in the culture broth. According to the procedure, εPLHCl was separated from 100 μL of the culture broth without any deproteinization. Figure 6b shows the chromatogram of a solution of the separated εPL species. Here, the separated εPLHCl was dissolved into 100 μL of water, although the analyte can be concentrated into a smaller volume of the solvent. The intensities of the impurity peaks and background signals were much smaller than those of the culture broth. The yield was ca. 95%.

The method has been successfully applied to the separation of lower-molecular-weight εPL, which was obtained by hydrolysis of the standard material. It has been shown that εPL species with \( n \geq 4 \) can be separated. Therefore, the method would be applicable to the separation of other polyamines or basic oligopeptides.

### 2-3 Colorimetric PPi determination and adenylation enzyme assay

The adenylation of metabolites is a ubiquitous reaction producing reactive metabolic intermediates. The εPL-synthetase contains the adenylation domain (A-domain), which activates the carboxyl groups of amino acid substrates with ATP, forming an aminoacyl-adenylate (aminoacyl-O-AMP) and PPi, according to the following reaction:

\[
\text{H}_2\text{NRCOOH} + \text{ATP} \rightleftharpoons \text{H}_2\text{NRCO-O-AMP} + \text{PPi}. \tag{1}
\]

Traditionally, the enzymatic activity has been measured by radioactive ATP-\(^{[32P]}\)PPi exchange assays. However, most facilities are not equipped to handle radioactive materials. Thus, we have developed a colorimetric method for the determination of PPi (Fig. 7).

First, a 20-μL aliquot of the sample solution is mixed with 200 μL of the 20 mM Na2MoO4, 0.6 M HCl, and 60% (v/v) acetonitrile (AN) solution to prepare an acetonitrile–water (AN-W) mixture. In this mixture, PPi forms a yellow 18-molybdopyrophosphate \([(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4–}\) anion, according to the reaction: \(\text{P}_2\text{O}_7^{4–} + 18\text{MoO}_4^{2–} + 36\text{H}^+ \rightarrow [(\text{P}_2\text{O}_7)\text{Mo}_{18}\text{O}_{54}]^{4–} + 18\text{H}_2\text{O}.\)

The sample solution, that is, the enzyme reaction mixture, would consist of ATP and AMP, as
described below. In the AN–W mixture, AMP does not give the colored heteropolytungstate species, which interferes with the present colorimetric PPi assay. On the other hand, ATP gives the 12-molybdophosphate \(
[(PO_4)Mo_{12}O_{36}]^{3–}\) anion through the hydrolysis of triphosphate ester to phosphate anion. However, the formation of the \([\text{PO}_4]\text{Mo}_{12}\text{O}_{36}\)\(^{3–}\) anion by ATP is much slower than that of the \([\text{P}_2\text{O}_7]\text{Mo}_{18}\text{O}_{54}\)^{4–} anion.

Second, \(4\ \mu\text{L}\) of the AN solution consisting of 50 mM bis(triphenylphosphoranylidene)ammonium chloride (BTPPACl) is added to the AN–W mixture to form the BTPPA⁺-[\(\text{P}_2\text{O}_7\text{Mo}_{18}\text{O}_{54}\)]\(^{4–}\) precipitate. The mixture is centrifuged, and the supernatant is removed. These operations should be completed within 10 min after preparing of the AN–W mixture so as to reduce the contamination of the BTPPA⁺-[\(\text{PO}_4\text{Mo}_{12}\text{O}_{36}\)]\(^{3–}\) precipitate.

Third, the BTPPA⁺-[\(\text{P}_2\text{O}_7\text{Mo}_{18}\text{O}_{54}\)]\(^{4–}\) precipitate is mixed with 220 \(\mu\text{L}\) of AN or propylene carbonate. The yellow BTPPA⁺-[\(\text{P}_2\text{O}_7\text{Mo}_{18}\text{O}_{54}\)]\(^{4–}\) salt dissolves into the organic solvent. After centrifugation, 200 \(\mu\text{L}\) of the supernatant is transferred into a microtube.

Fourth, 20 \(\mu\text{L}\) of the 0.5 M ASC, 2 M HCl, and 50 mM AN solution is added to the organic solution. In the organic solution, the \([\text{P}_2\text{O}_7]\text{Mo}_{18}\text{O}_{54}\)^{4–} anion is reduced to the 8-electron reduced species, which renders the solution blue. The reaction would terminate within around 10 min. As shown in Fig. 8, the intensity of blue of the reaction mixture increased with the concentration of PPi in the sample solution. When the sample solution contains ATP, the BTPPA⁺-[\(\text{P}_2\text{O}_7\text{Mo}_{18}\text{O}_{54}\)]\(^{4–}\) precipitate may contaminate slightly into the organic solution. However, the reduction of the \([\text{PO}_4]\text{Mo}_{12}\text{O}_{36}\)^{3–} anion is much slower than that of the \([\text{P}_2\text{O}_7]\text{Mo}_{18}\text{O}_{54}\)^{4–} anion. The slow reduction of the \([\text{PO}_4]\text{Mo}_{12}\text{O}_{36}\)^{3–} anion may be due to isomerization between the \(\alpha\)- and \(\beta\)-forms of the Keggin structure. Thus, a 10 mM level ATP would not interfere with the PPi colorimetric assay significantly.

The colorimetric PPi microassay can be advantageously applied to the assay of the A-domains, such as rPls-AT and ORF 19. An enzyme reaction mixture consisting of 50 mg/L enzyme, 5 mM MgCl₂, 5 mM ATP, and 50 mM Tris-HCl (pH 8.0) was incubated at 30°C for 30 min. To depress the reverse reaction of Eq. (1), the enzyme reactions were performed in the presence of 32 mM \(\text{NH}_2\text{OH}\), which reacts irreversibly with acyl-O-AMP to produce a hydroxamate derivative:

\[
\text{H}_2\text{NRCO-O-AMP + NH}_2\text{OH} \rightarrow \text{H}_2\text{NRCO-NHOH + AMP}. (2)
\]

In fact, HPLC/ESI-MS analysis proved the production of the hydroxamate derivatives. Also, it was confirmed that orthophosphate was not produced in the enzyme reaction mixtures by a spectrophotometric assay. As shown in Fig. 9, the enzyme reaction mixtures with the substrates gave a positive blue color. In contrast, the negative controls showed no color. These results coincide with those by ATP-[\(\text{P}\)]PPi exchange assay in previous studies.

### 3 Bioelectroanalysis Method for the Evaluation of ePL as an Additive for an Enzyme Reaction Mixture

#### 3-1 Enzyme-stabilizing effect

Figure 10 shows the cyclic voltammogram (CV) of the 50 mM phosphate buffer (pH 6.0) containing 0.1 M glucose, 1 g/L GOD, and 50 \(\mu\text{M}\) hydroquinone (HQ) at 25°C with a glassy
A time-dependent decrease in enzyme, the inactivation process of GOD can be followed by the first-order kinetics of the inactivation:

$$I_l \propto \sqrt{C}$$

where $k$ is the first-order rate constant of Eq. (3) and $I_{l,0}$ the $I_l$-value at $t = 0$. By fitting of Eq. (4) to the $I_l$ vs. $t$ plot in Fig. 12, the Arrhenius plot gave a straight line, indicating that the same inactivation mechanism can be assumed in the temperature range. From the slope, the Arrhenius activation energy was calculated to be $205 \pm 13$ kJ mol$^{-1}$, which is comparable with a reported value obtained by the conventional spectrophotometric enzyme assay. 

By the addition of $\varepsilon_{PL}$, the $I_{l,0}$ was not changed significantly, indicating that $\varepsilon_{PL}$ has no influence on the GOD reaction with the charge neutral oxidant, 1,4-benzoquinone. However, in the presence of 1 g/L $\varepsilon_{PL}$ at 70°C, the $k$-value was determined to be $k = (1.8 \pm 0.9) \times 10^{-4}$ s$^{-1}$, which is smaller than that in the absence of $\varepsilon_{PL}$. At $pL_{GOD} (= 4.2) < \varepsilon_{PL} < pL_{K_{a,PL}}$ (pI$_{GOD}$ being the isoelectric point of GOD), GOD molecule would carry negative charges on the surface, and a polycationic $\varepsilon_{PL}$ species would adsorb onto the enzyme surface. Although the kinetic measurement was performed at a higher concentration of $\varepsilon_{PL}$, the $k$-value did not change significantly. On the other hand, the stabilizing effect decreased with the concentration of $\varepsilon_{PL}$ when the concentration was less than 1 g/L. The adsorption of $\varepsilon_{PL}$ onto the GOD may be saturated at 1 g/L level. It is noted that 1 g/L monomeric L-lysine did not decrease the $k$-value significantly. Also, no significant decrease in $k$ was observed with 1 g/L of well-known stabilizers, glycerol, trehalose, and ammonium sulfate, which are usually added at much higher concentrations.

As shown by black circles in Fig. 12, the Arrhenius plot gave a straight line also in the presence of 1 g/L $\varepsilon_{PL}$. The slope was nearly equal to that in the absence of $\varepsilon_{PL}$, indicating that the decrease in $k$, that is, the increase in the activation Gibbs energy for the reaction of Eq. (3), can be explained by the decrease in the activation entropy without an increase in the activation enthalpy. Thus, the stabilizing effect by $\varepsilon_{PL}$ may be ascribed to the formation of a polyanion complex of GOD, by which the local disorder of the enzyme surface may be reduced in the inactivation process.

Fig. 10 Cyclic voltammogram (CV) of the 0.1 M glucose, 1 g/L GOD, 50 $\mu$M hydroquinone, and 50 mM phosphate buffer (pH 6.0) at 25°C with a glassy carbon electrode. Scan rate, 0.002 V s$^{-1}$.

Fig. 11 Time-dependence of the limiting current ($I_l$) in the 0.1 M glucose, 1 g/L GOD, 50 $\mu$M hydroquinone, and 50 mM phosphate buffer (pH 6.0) at 70°C. Solid line, calculation curve by Eq. (4) with $k = 6.6 \times 10^{-4}$ s$^{-1}$.

Fig. 12 Arrhenius plots of the rate constant ($k$) for the thermal inactivation of GOD in 50 mM phosphate buffer (pH 6.0) in the absence (○) and presence (●) of 1 g/L $\varepsilon_{PL}$.
The kinetic measurement was also conducted in the presence of 1 g/L of other polyamines and polyammonium salts. Polyallylamine and polyethyleneimine formed a suspended substance with GOD. α-Polylysine, polyethyleneimine 80% ethoxylated, poly[oxethylene(dimethylamino)propylene-(dimethylamino)ethylene dichloride], and quaternized poly[bis-(2-chloroethyl)ether-alt-1,3-bis[3-(dimethylamino)propylene]urea] did not decrease the $k$-value, as low as the decrease caused by εPL. At present, it is not clear why εPL stabilizes GOD more effectively than the other cationic polymers. However, the polyion complex of GOD with εPL may be stable in the solution much more than those with other cationic polymers.

### 3-2 Promotion effect by εPL on the GOD reaction with [Fe(CN)$_6$]$^{3–}$ as an oxidant

The electrostatic interaction between enzyme and reactant should influence the enzymatic reaction rate. For example, the GOD-catalyzed oxidation of glucose at pH > $p_I_{GOD}$ tended to exhibit a faster reaction rate with more positively or less negatively charged oxidants. On the other hand, the GOD enzymatic reaction with [Fe(CN)$_6$]$^{3–}$, which possesses multiple negative charges, takes place at a lower reaction rate compared to other oxidants.

Figure 13a shows the CV for the 0.2 M glucose, 5.1 μM GOD, 100 μM K$_3$[Fe(CN)$_6$], and 50 mM phosphate buffer (pH 6.0) in the absence of GOD and glucose, indicating that the GOD reaction with [Fe(CN)$_6$]$^{3–}$ is not fast enough to confirm a catalytic current. When εPL was added to the solution, an S-shaped $I$-$E$ curve with a limiting current was observed (Fig. 13b). The limiting current can be attributed to the [Fe(CN)$_6$]$^{4–/3–}$-mediated bioelectrocatalytic current for the oxidation of glucose by GOD. The results clearly indicate that the rate of the GOD reaction with [Fe(CN)$_6$]$^{3–}$ was increased remarkably by the presence of εPL. Surprisingly, the estimated value for the bimolecular rate constant between GOD and [Fe(CN)$_6$]$^{3–}$ was 10000-fold larger than that in the absence of εPL. The increment in the reaction rate, that is, the promotion effect by εPL, was observed remarkably at $p_{L_{GOD}} < pH < pK_{a,PL}$. The effect was not observed significantly at pH < $p_{L_{GOD}}$, and was reduced with increasing pH at pH > $pK_{a,PL}$. Therefore, the effect can also be explained by the polyion complex formation of GOD with εPL, by which the enzyme surface is charged positively. Thus, the electrostatic repulsion between GOD and [Fe(CN)$_6$]$^{3–}$ would be reduced, so that the electron-transfer occurred effectively between them.

The mechanism seems to be simple. However, many other cationic polymers tested formed an insoluble polyion complex with [Fe(CN)$_6$]$^{4–/3–}$ and/or GOD. Thus, no such remarkable effect has been observed with other cationic polymers. Also, a structural study on the polyion complex is needed for further understanding of the effect.

### 4 Conclusions

Our colorimetric εPL microassay can be applied advantageously to the screening of εPL-synthetase. Our separation method has been successfully applied to the separation of εPL from 100 μL level culture broth. The chemical separation method is useful in the pretreatment of the biological samples more advantageously than the conventional column separation method. These methods are based on the precipitation of polycationic εPL with the hydrophobic anions, and would also be useful in studies of other polyamines and basic oligopeptides. Our colorimetric PPi microassay can be applied to the assay of adenylation enzyme. The method is much more convenient than the conventional radioactive ATP-[32P]PPi exchange assay. The measurements of bioelectrocatalytic current in the presence of εPL showed new uses of εPL as the enzyme stabilizer and the enzyme reaction enhancer.

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