Purification and the Catalytic Site Residues of *Pseudomonas fragi* Lipase Expressed in *Escherichia coli*

Tae-Ryun Kim* and Chul-Hak Yang

Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea
**Department of Chemistry, College of Natural Sciences, Duksung Women's University, Seoul 132-714, Korea*
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The *P. fragi* lipase overexpressed in *E. coli* as a fusion protein of 57 kilodalton (kDa) has been purified through glutathione-agarose affinity chromatography by elution with free glutathione. The general properties of the purified GST-fusion protein were characterized by observing absorbance of released *p*-nitrophenoxide at 400 nm which was hydrolyzed from the substrate *p*-nitrophenyl palmitate. The optimum condition was observed at 25 ℃, pH 7.8 with 0.4 μg of protein and 1.0 mM substrate in 0.6% (v/v) TritonX-100 solution. Also the lipase was activated by Ca^{+2}, Mg^{+2}, Ba^{+2} and Na^{+} but it was inhibited by Co^{+2} and Ni^{+2}. pGEX-2T containing *P. fragi* lipase gene as expression vector was named pGL19[1] and used as a template for the site-directed mutagenesis by sequential PCR steps. A Ser-His-Asp catalytic triad similar to that present in serine proteases may be present in *Pseudomonas* lipase. Therefore, the PCR fragments replacing Asp217 to Arg and His260 to Arg were synthesized, and substituted for original fragment in pGL19. The ligated products were transformed into *E. coli* NM522, and pGEX-2T harboring mutant lipase genes were screened through digestion with *Xba*I and *Stu*I sites created by mutagenic primers, respectively. No activity of mutant lipases was observed on the plate containing tributyrin. The purified mutant lipases were not activated on the substrate and affected at pH variation. These results demonstrate that Asp217 and His260 are involved in the catalytic site of *Pseudomonas* lipase.

Introduction

Lipases are widely distributed in various animals, plants and microorganisms. In particular, lipases from microorganisms are important enzymes for practical purposes such as food additives, medicines, clinical reagents, detergent, and so on. Many psychrophilic bacteria such as *Pseudomonas, Achromobacter, Flavobacterium, Alcaligenes, Escherichia,* and *Aerobacter* possess a lipolytic enzyme system that releases short-chain fatty acids during the hydrolysis of fat which are responsible for abnormal flavors.[2] Recent studies on fungal lipases have been shown that lipases exhibit two kinds of substrate specificity; those are, "positional specificity" for the ester bonds of triacylglycerides and "fatty acid specificity" for the fatty acid components of triacylglycerides. Mammalian lipases exhibit a high degree of selectivity. For example, rabbit pancreatic lipase shows the hydrolysis of long-chain triacylglycerides at 1 and 3 positions into more polar, free fatty acids and 2-monoacylglycerides.[3] Porcin pancreatic lipase, rabbit gastric lipase and human gastric lipase give stereo selective hydrolysis of 1 or 3 position.[4] On the other hand, extracellular bacterial lipase from *Staphylococcus aureus* hydrolyzes tri-, di- and monooleoylglycerol. This lipase lacks positional specificity, and hydrolyzes substrate rapidly to free fatty acids and glycerol without accumulation of intermediary products.[5] Lypolytic enzyme has been defined as "long chain fatty acid ester hydrolase" or as "any esterase capable of hydrolyzing esters of oleic acid".[6] Although such definitions have utility, they only indirectly acknowledge the more fundamental distinction between lipases and esterases, the involvement of a lipid-water interface in the catalytic process.

In 1967, Mencher and Alford reported on research with purified *P. fragi* lipase enzyme.[7] The purification and properties of the same lipase was published by Lu and Liska.[8] These procedures are very complicated and tedious. Here we are reporting effective isolation of the lipase from *P. fragi* in *E. coli* using the GST gene fusion system by affinity chromatography. In fact such fusion proteins are soluble and easily purified from lysed cells under nondenaturing conditions by absorption with glutatione-agarose beads, followed by elution in the presence of free glutathione. Also, fusion proteins expressed using pGEX2T or pGEX3X contain an amino acid sequence between the GST carrier and the foreign polypeptide that is cleaved by the site-specific protease thrombin or blood coagulation factor Xa. Two alternatives are available for cleavage of fusion protein: to conduct the cleavage reaction on the pure fusion protein in solution,[9] or to cleave the foreign polypeptide from GST while it is still bound to glutathione-agarose beads.[10] Finally, the foreign protein can be obtained from fusion protein by protease.

In according to analysis of three dimensional structure of some lipases, the active centers were found as buried inside. They have the trypsin-like catalytic triads (Asp-His-Ser) at the active site which is analogous to those of serine proteases.[11~18] However, no crystal structure of bacterial lipases are known. Only a few reports of the primary structures on *Pseudomonas* species have been reported.[19~21] Therefore, the relationship between the structure and the function of the bacterial enzyme is not known yet. Comparison of the primary structures of these lipases may provide the significant imformation about function of the enzyme. In addition to Ser, the sequence alignments of Asp and His from various lipases were introduced by Chihara-Siomi *et al.*, too.[22] Therefore, identification of these residues as catalytic sites is reasonable.

In this study, we report purification and characterization of lipase from *P. frgi* IFO-12049 as the fusion protein in *E. coli.* In order to identify the active center, we report the

site-directed mutagenesis replacing aspartate and histidine supposed as catalytic sites with arginine, respectively.

Materials and Methods

Materials. Pepton, trypton and bacto agar were purchased from Acumedia. D-cycloserine, tributyrin, ampicilline, protein marker, BSA, sodium dodecylsulfate, acrylamide, bisacrylamide, TEMED, glutathione, glutathione-agarose, thrombin and *p*-nitrophenyl palmitate were purchased from Sigma. Restriction endonucleases and T4 DNA ligase were purchased from KOSCO. IPTG and Magic PCR product purification system were purchased from Promega. TritonX-100, 2-mercaptoethanol, ethanol, chloroform and DMSO were purchased from Merck. pGL19, recombinant plasmid DNA containing *P. fragi* lipase gene, was used as a template for site-directed mutagenesis.

Purification of fusion protein. All operations, unless stated otherwise, were carried out at 4 ℃. 2 g of *E. coli* NM522 cells induced by IPTG, which was grown aerobically in LB medium, was suspended with 20 mL phosphate buffered-saline (PBS) (8 g of NaCl, 0.2 g of KCl, 1.15 g of $Na_2PO_4 \cdot 7H_2O$ and 0.2 g of KH_2PO_4 per 1 liter). The suspension was sonicated for 2 min (20 sec pulsed) and 10% Triton X-100 was added to 1% and gently mixed. After centrifugation at 12000×g for 20 min, the supernatants were collected.

GST-lipase was purified through glutathione agarose affinity column. The glutathione-agarose affinity column (0.7×8 cm) was washed with 20 mL ice-cold PBS. The supernatants were loaded to the affinity column and desired protein was bound. It was washed again with 20 mL PBS. GST-lipase was eluted with 10 mL of 10 mM free glutathione (flow rate: 0.1 mL/min). Each fraction was collected. The lipase activity of each fraction was assayed with *p*-nitrophenyl palmitate as a substrate.

Cleavage of Fusion Protein by Thrombin. Purified fusion protein was cleaved at the juction between GST and lipase by incubation at 25 ℃ in elution buffer containing 150 mM NaCl, 2.5 mM $CaCl_2$ and 100 ng of human thrombin.

Protein Assay. Protein concentration was determined by the method of Lowry *et al.*[23] Bovine serum albumin (BSA) was used as the standard.

IPTG Induction and SDS-PAGE. The overproduction of fusion protein was analyzed on SDS-PAGE. *E. coli* NM522 carrying lipase gene was inoculated into LB media containing 50 g/mL of ampicillin and grown at 37 ℃ to 0.7 of optical density (A_{600} 0.7). IPTG was added to the final concentration of 1 mM and the incubation was continued. 1 mL of cultures were collected every 1 hr and microcentrifuged at 5,000×g for 1 min. The pellet was resuspended in 100 μL of SDS-sample buffer and heated for 2 min in boiling water prior to loading.

SDS-discontinuous PAGE was performed according to the method of Laemmli[24] using 0.75 mm slab gel which was composed of resolving gel (pH 8.8) of 10% (w/v) polyacrylamide and stacking gel (pH 6.8) of 3% polyacrylamide. After electrophoresis, staining of protein was carried out either employing Coomassie brilliant blue or the silver stain method.[25]

Enzyme Assay. The reaction mixture of 1.0 mM *p*-nitrophenyl palmitate containing 0.6% Triton X-100, 50 mM Tris-Cl buffer (pH 8.0), was prepared by heating in microwave oven for 30 sec and cooled at room temperature until the mixture was optically clear.[26,27] An aliquot of 10 μL of enzyme solution was added to test tube containing 1 mL of the reaction mixture. The reaction was carried out by incubating at 25 ℃ for 10 min and terminated by immersing the reaction mixture into ice-water slurry. The hydrolysis of *p*-nitrophenyl palmitate was determined by measuring the absorbance at 400 nm. One enzyme unit is defined as 1 nmol of *p*-nitrophenol released mL^{-1} min^{-1}.

(A)

Start primer

5′-CCAAAATCGGATCTGGTTCCGCGT

Halt primer

5′-CAGATCGTCAGTCAGTCACGATGA

(B)

Mutagenic primers

Asp217 -----> Arg217

D1

5′-CTCAACCTGCTCAGGCCTTTGCACAAT

StuI

D2

5′-ATTGTGCAAAGGCCTGAGCAGGTTGAG

His260 -----> Arg260

H1

5′-TACCCTCTAGACCGCCTCGACA

XbaI

H2

5′-TGTCGAGGCGGTCTAGAGGGTA

Figure 1. Primers designed for site-directed mutagenesis by sequential PCR. (A) PCR primers; start and halt primers have complementary sequence with pGEX2T to the left and right at multicloning sites, respectively. These primers creat *Bam*HI and *Hin*dIII sites during PCR when pGL19 was used as a template. (B) Mutagenic primers; D and H primers were changed GAC (Asp217) and CAC (His260) to AGG and CGC (Arg). The restriction sites of *Stu*I and *Xba*I were created without changing of other amino acids. Solid spellings are designated the replacement to other nucleotides and underline shows restriction and mutagenic sites.

Characterization of *P. Fragi* Lipase. Optimal pH level was determined by using 50 mM Tris-Cl buffer system. The pH range can only be determined in above pH 7.4 because the color of *p*-nitrophenol disappeared in pH below 7.4. The effect of incubation time was determined. Temperature dependence was measured by incubating the reaction mixture at the desired temperature for 10 min. The effects of cations were observed with Ca^{++}, Mg^{++}, Ba^{++}, Na^{+}, Co^{++} and Ni^{++} at several different concentrations.

Site-Directed Mutagenesis by PCR. Six PCR primers are shown in Fig. 1. Start and halt primers create

*Bam*HI and *Hin*dIII sites, respectively. Each D and H of mutagenic primers contains *Stu*I and *Xba*I restriction ezyme sites, respectively. pGL19 was used as template for site-directed mutagenesis by squential PCR steps. The PCR mixture contains 10 ng of pGL19, 100 pmol of each primer, 50 mM Tris-Cl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 units of *Taq* DNA polymerase in final volume of 100 μL. This mixture was covered with 100 μL of paraffin oil to prevent evaporation. Annealing was performed at 50 ℃ for 1 min, extension at 72 ℃ for 30 sec and denaturation at 93 ℃ for 1 min with 20 cycles at both the first and second amplifications. Denaturation of the first cycle was done for 5 min and extension of the last cyle was done for 10 min. For substitution Asp217 to Arg, the first steps were perfomed by start and D2 primers and D1 and halt primers, respectively. Each amplified product was prepared for second steps. The same method was used in replacement His260 to Arg. The purification of PCR product of the first step was performed by Magic PCR product purification system shown below.

Purification of PCR Products. Each completed PCR reaction was transfered the aqueous (lower) phase to a clean microcentrifuge tube. The presence of too much mineral oil in the sample can lead to a decreased yield in the PCR product purification. 100 μL of PCR product was mixed with 100 μL of direct purification buffer (50 mM KCl, 10 mM Tris-Cl (pH 8.8), 1.5 mM $MgCl_2$ and 0.1% TritonX-100). After adding 1 mL of Magic PCR preps resin, the mixture was vortexed briefly. The Magic minicolumn was loaded with the resin/DNA mix and washed with 2 mL of 80% isopropanol. The minicolumn was attached microcentrifuge tube and centrifuged at 12000×g for 20 sec to dry the resin and transfered to a new microcentrifuge tube. After incubation with 100 μL of water for 1 min, it was centrifuged at 12000×g for 20 sec to elute the bound DNA fragment. The purified DNA was stored at 4 ℃.

Subcloning. The original pGL19 was digested with *Bam*HI, *Hin*dIII, *Nde*I and *Sma*I and ligated with the PCR fragment amplified by sequential PCR steps. The digested pGL19 and PCR fragment were ligated with molar ratio of 1 : 5 and transformed into *E. coli* NM522 by the method of Maniatis, *et al.*[28]

Results and Discussion

The overproduction of lipase was successful by the expression vector pGEX2T. This vector system has following advantages; 1) the *tac* promoter for inducible and high-level expression, 2) an internal $lacI^q$ gene for host independence, 3) the cleavage site for thrombin to allow the release of desired protein from fusion product, 4) easy purification using affinity chromatography, 5) mild elution conditions, to maintain functional activity. The GST-lipase fusion protein of 57 kDa was purified by affinity column chromatography using glutathion-agarose beads (Fig. 2, A). However, the majority of the fusion protein was formed in inclusion bodies, and only a small portion was soluble. Although the majority of expressed fusion protein was insoluble, the soluble portion was enough to characterize the enzymatic properties. This insolubility is a frequent characteristic of foreign proteins expressed in *E. coli*[29] and little is known about the factors

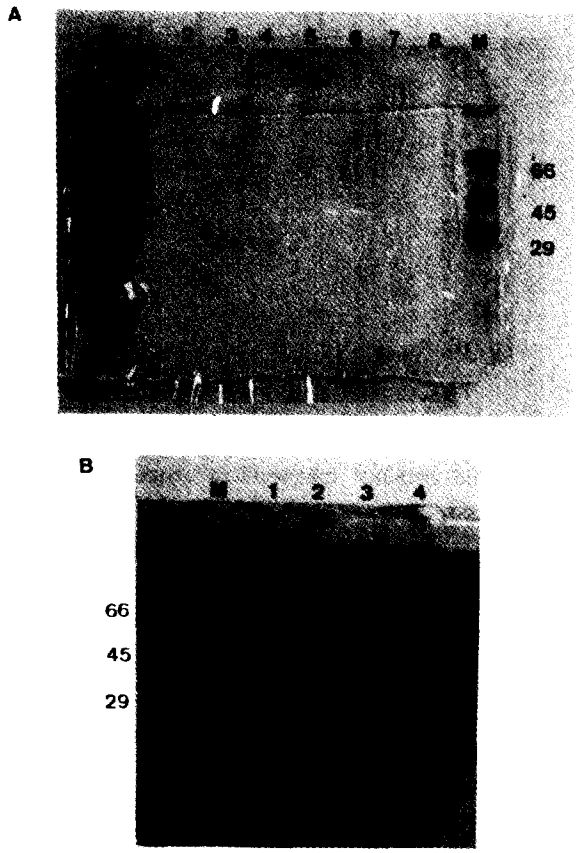

Figure 2. SDS-PAGE analysis. (10% acrylamide) (A) After affinity column. Lanes: M, protein size marker; 1, crude cell extract, NM522/pGL19; 2-9, eluents. The molecular mass markers were indicated on the right. (B) Site-specific proteolysis using thrombin. Lanes: M, protein size marker; 1, eluent through affinity column; 2, after thrombin treatment; 3, supernatant of centrifuged lane 2; 4, pellet of cetrifuged lane 2.

responsible for insolubility.[30] In this experiment, the insolubility of fusion protein seems to be associated with the high hydrophobicity of lipase. According to the report, the elimination of hydrophobic region of ligand protein greatly increased stability and/or solubility of fusion protein, in GST fusion system.

Using the recognition site of thrombin at the junction between GST and lipase,[9] native lipase could be obtained after thrombin treatment. However, the precipitate was formed after thrombin treatment. SDS-PAGE revealed that the precipitate formed was insoluble portion of lipase (Fig. 2, B). The foreign polypeptide may become insoluble when it was cleaved from the GST-carrier. It has been reported that some purified lipases are unstable in aquatic solution state. The stability of lipase of *P. fragi* decreases with purification.[8] Because of reaction conditions of thrombin treatment and its hydrophobicity, the native enzyme may be very unstable. So a lipase fused with a protein was obtained and needed for study of its characteristics.

For the fundamental distinction between lipases and esterases is the involvement of lipid-water interface in catalytic process.[31] The micellar mixture of *p*-nitrophenyl palmitate is better subtsrate than water-soluble tributyrin in measuring

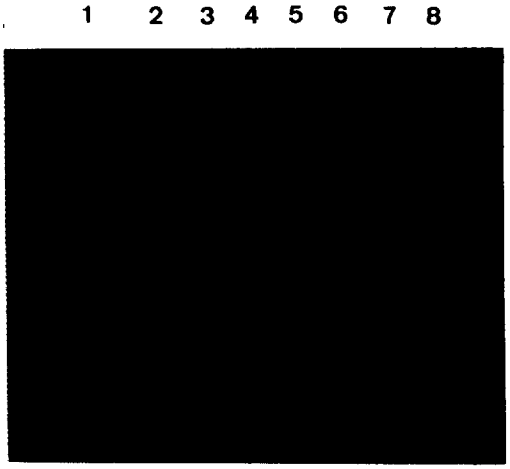

Figure 3. The amplified fragments by sequential PCR steps. (2% agarose gel electrophoresis) Lanes: 1, 8, DNA size marker of pUC18 digested with *Sau*3AI; 2, amplified fragment by D1 and halt primers; 3, amplified fragment by start and D2 primers; 4, second PCR product of 2 and 3; 5, amplified fragment by H1 and halt primers; 6, amplified fragment by start and H2 primers; 7, second PCR product of 5 and 6.

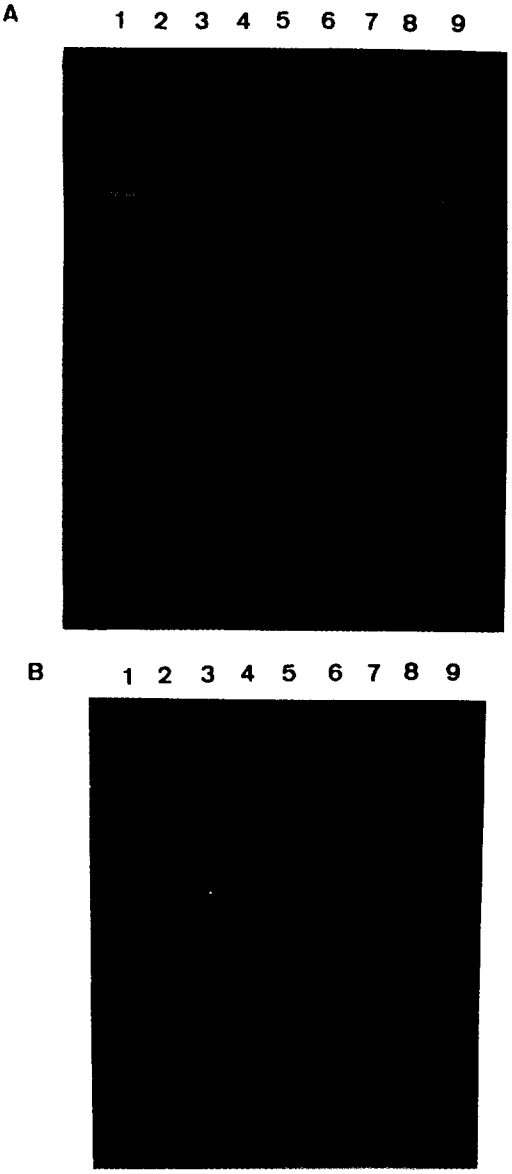

Figure 4. Identification of pGLD5 and pGLH8. (0.7% agarose gel electrophoresis) pGLD5 and pGLH8 are pGEX2T haboring mutant genes substitued Asp to Arg and His to Arg, respectively. (A) pGLD5. Lanes: 1, 9, λ DNA marker digested with *Eco*RI and *Bam*HI (λ DNA/*Eco*RI and *Bam*HI); 2, pGL19/*Bam*HI and *Hin*dIII; 3, pGLD5/*Bam*HI; 4, pGLD5/*Hin*dIII; 5, pGLD5/*Bam*HI and *Hin*dIII; 6, pGL19/*Stu*I; 7, pGLD5/*Stu*I; 8, pGLD5/*Eco*RI. (B) pGLH8. Lanes: 1, 9, λ DNA/*Eco*RI and *Bam*HI; 2, pGL19/*Bam*HI and *Hin*dIII; 3, pGLH8/*Bam*HI; 4, pGLH8/*Hin*dIII; 5, pGL 19/*Xba*I; 6, pGLH8/*Xba*I; 7, pGLH8/*Bam*HI and *Hin*dIII; pGLH8 /*Eco*RI.

lipase activity. It was reported that trypsin-like catalytic site of lipase is buried under a loop, called 'lid', therefore inaccessible to solvents,[13,14,32,33] and this loop seems to have a role in interfacial activation of lipase at water-lipid interface. If the amino acid responsible for the flexibility of joint of the loop is identified, the catalytic triad at active site will be exposed permanently to solvents and substrates by replacing it with another amino acid carrying bulky residue. It has been pointed that heterogeneity and temporal change of reaction mixtures were two major complications which made general concept of lipase action ambiguous.[34] But the lipase of *P. fragi* was not a structural domain, as the lid, when it was compared with sequence homology of C-teminal amino acids of pancreatic lipases.[32] This lipase may be exposed permanently to solvents and substrates. The substrate *p*-nitrophenyl palmitate offers a better model of lipolysis in that it is not water soluble and mixed micelle is formed with the addition of detergent such as Triton X-100. The general properties of the purified GST-lipase expressed in *E. coli* were characterized. The activities of the lipase increased linearly when it were incubated for 10 min at 0.2 and 0.4 μg protein, respectively. The pH dependence of the enzyme was examined at the range between 7.4 and 8.8 and showed a relatively high reaction rate with the optimal pH value around 7.8. This result is in agreement with the work of Lu and Liska.[8] However, it is somewhat different from that of Nashif and Nelson who reported the optimal pH of 7.0.[34] Interestingly, optimal pH of 8.6 to 8.7 was reported by Mencher and Alford for the lipase of *P. fragi*.[7] These variation may be due to GST-lipase fusion protein system and difference in substrate and assay procedure employed. Temperature is an important factor which affects both reaction rate and physical state of the substrate mixture. In this experiment the observed reaction rate was increased up to 25 ℃ and then dropped sharply above 40 ℃. Cations generally form complexes with ionized fatty acids, changing their solubility and behavior at lipid-water interface. It has been pointed out that in lipase-catalyzed hydrolysis of triacylglycerides, the release of fatty acids to the medium is frequently the rate-determining step. Therefore, the addition of divalent ion such as Ca^{++} and Mg^{++} has exerted some activation effect. However interestingly the effects of cations depend on particular lipase. Thus the effects probably also involve

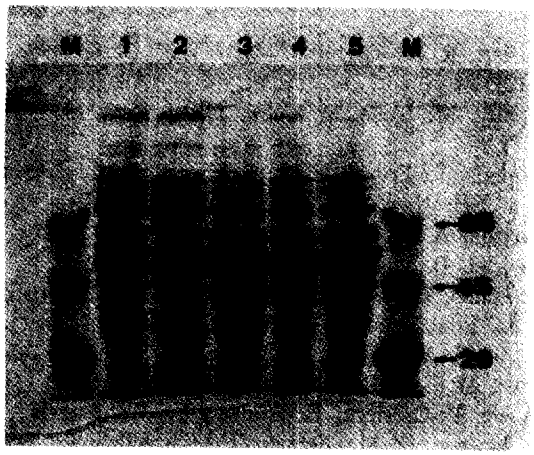

Figure 5. SDS-PAGE analysis of overexpressed mutant lipases. (10% acrylamide) Lanes: M, molecular weight marker protein; 1, NM522 cell only (control); 2-5, NM522/pGEX2T, NM522/pGL 19, NM522/pGLD5 and NM522/pGLH8, after induction, respectively. These lipases were induced using IPTG and lipases of mutant type were overproduced as wild type.

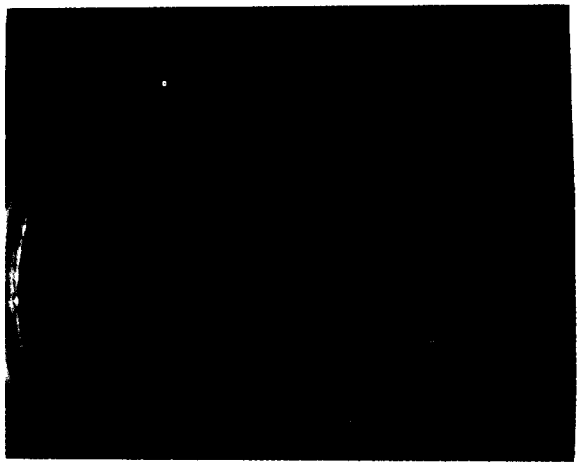

Figure 6. The comparison of activity of wild and mutant type lipases. The activity of these lipases was determined on tributyrin-agar plate containing IPTG as clear zone. *E. coli* NM522, NM 522/pGEX2T and NM522/pGL19 were used as control. NM522 /pGLD5 and pGLH8 did not apear as clear zones on plate.

the catalytic site in some case.[35] At this experiment, Co^{++} and Ni^{++}, of the cations tested, exibited strong inhibition on the activity of GST-lipase at 20 mM, whereas Ca^{++}, Ba^{++}, Na^{+} and Mg^{++} strongly activated the reaction. Especially, in the case of Na^{+}, the enzyme activity was increased to five times in comparison to control (without cation) at 100 mM.

Lipase of *P. fragi* is composed of 277 amino acids and it contains 12 of histidines and 14 of aspartic acids. The catalytic center of *P. fragi* is supposed to be Ser83, Asp217 and His260 based on the currently known informations Assuming that *P. fragi* lipase has a trypsin-like catalytic center (Asp-His-Ser) observed in other lipases,[22] Asp-His-Ser would be that of catalytic center. To determine whether Asp217 or His260 is involved in catalytic center, we performed site-directed mutagenesis by PCR. This PCR method has many advantages to the normal site-directed mutagenesis. We designed the mutagenic primers for sequential PCR steps. For screening of mutant lipase, these primers were created to contain restriction sites without changing other amino acids except mutagenic site (Fig. 1). In this method, after obtaining fragments carrying mutant lipase genes (Fig. 3), mutant fragments for original fragment in pGL19 were substitued. These ligated genes were transformed into *E. coli* NM522 and screened by restriction enzymes (Fig. 4) and IPTG induction (Fig. 5), respectively. These plasmids were isolated and designated as pGLD5 and pGLH8. The cells harboring the pGLD 5 and pGLH8 had no activity on tributyrin agar plate containing IPTG (Fig. 6). After IPTG induction mutant proteins were purified and their activities were examined. The activity of purified mutant proteins was strongly decreased, comparing to that of wild type. Also, no change in activity was observed by changing pH. In view of the results so far achieved, changing His260 or Asp217 to a arginine produced inactive lipase. On the basis of above results, it is concluded that Asp217 and His260 exist in catalytic center of *P. fragi*.

In order to determine the remaining Ser82, it could be replaced by another amino acids. It is assumed to be important as an active site.

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A Study on the Complexation of Copper(II) Ion with 2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol in Aqueous Solution

Kyung Hee Hong, Eun Jong Ha, and Kyu Sun Bai*

Department of Chemistry, Soong Sil University, Seoul 156-743, Korea

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The complex formation from Cu(II) ion and 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bistris) in aqueous solution has been studied potentiometrically and spectrophotometrically. Bistris (L) coordinates to Cu(II) as tridentate. The complex CuL^{2+} undergoes deprotonation in neutral and basic media. The deprotonated complexes involve metal-alcoholate coordinate bond in stable chelate structures.

Introduction

Tris(hydroxymethyl)aminomethane (Tris) and bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bistris) are used extensively as buffer materials in the studies of biochemical systems. Bistris was first synthesized in 1966.[1] The thermodynamic parameters of Bistris have been reported.[2~4] The stability constants of complexes formed by the combination of the neutral Bistris and some transition and nontransition metal ions have been determined by potentiometric and spectrophotometric methods by Scheller *et al.*[5] The complex formation from Bistris and alkaline earth metals have been studied by Sigel *et al.*[6]

However, the complex formation between Bistris and metal ions where the hydroxyl proton of the coordinated ligand is displaced by the metal has not been investigated. In the present study, the various complexes formed from Bistris and Cu(II) ion in wide pH ranges have been studied potentiometrically and spectrophotometrically.

Experimental

Bistris used in this study was the '99+ %' grade from Aldrich Chemical Company. It was dried for 24 hours at 80 ℃ before use. All the other chemicals used were of reagent grade. The Cu(II) nitrate solution was standardized by complexometry. Twice-recrystalized potassium nitrate was used to maintain ionic strength.

In all experiments the hydrogen ion activity was measured with Orion Research EA-940 expandable ion analyzer and Ross 81-01 combination electrode. The pH meter was calibrated with phthalate and phosphate buffers. The hydrogen ion concentration was obtained from the measured pH by using the activity coefficient at the ionic strength used here.[7] The hydroxide ion concentration was obtained by using the value of 13.78 for pKw of water.[8]

Solutions of Bistris and hydrochloric acid were titrated with standard sodium hydroxide solution in the presence and absence of Cu(II) ion. All titrations were carried out at 25 ℃ using 50 mL test solutions. The ionic strength was kept at 0.1 M with KNO_3. The titrant was 0.966 M NaOH. A 2-mL Gilmont buret was used. The electronic absorption spectra were taken with Perkin Elmer Model 551S spectro-