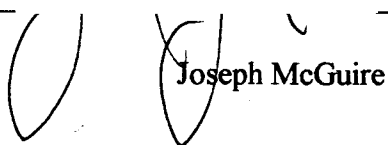AN ABSTRACT OF THE THESIS OF

Qiurong Xu for the degree of Master of Science in Bioresource Engineering presented on

May 19, 1997. Title: Structural Effects on Enzymatic Activity of Bacteriophage T4

Lysozyme upon Adsorption to Colloidal Silica.

Redacted for Privacy

Abstract approved by :___

Joseph McGuire

The enzymatic activities of bacteriophage T4 lysozyme mutants with different structural

stabilities were measured in the presence and absence of colloidal silica nanoparticles using

a spectrophotometric technique and an agar plate bioassay. Spectrophotometric technique

is based on the continuous reading of the change in turbidity of a bacterial suspension,

while agar plate bioassay is based on the comparison of the clear zone width developed by

T4 lysozymes. Wild type (isoleucine at position 3) T4 lysozyme along with two structural

stability mutants Ile 3 → Cys and Ile 3 → Trp were produced from *Escherichia coli* cells

bearing the desired lysozyme genes. The three lysozyme variants differed by a single

amino acid residue at position three. Each substitution resulted an altered structural

stability, quantified by a difference in free energy of unfolding relative to wild type (-2.8

Kcal/mol and 1.2 Kcal/mol for Ile 3 → Trp mutant and Ile 3 → Cys mutant, respectively).

Two different substrates *E. coli* cell walls and *Micrococcus lysodeikticus* cell walls, were

used to measure the activity of each free lysozyme and lysozyme-particle complexes. For

a ratio of 1:1 protein to particle with a 90 minute adsorption time, all the T4 lysozyme

mutants lost part of their enzymatic activity. The extent of enzymatic activity reduction is related to the stabilities of the proteins. The mutants with lower stability had lower enzymatic activity and a greater tendency to lose their enzymatic activity upon adsorption.

Structural Effects on Enzymatic Activity of Bacteriophage T4 Lysozyme
upon Adsorption to Colloidal Silica

by

Qiurong Xu

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APPROVED:

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Chair of Department of Bioresource Engineering

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Qiurong Xu, Author

ACKNOWLEDGMENT

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CHAPTER 1

INTRODUCTION

The study of protein behavior at a solid-liquid interface is driven by the need for this knowledge in many fields including bioprocess and biomedical technology. Any process involving a surface and a protein solution is likely to be influenced by protein adsorption at the interface (Horbett et al., 1987). In the area of bioprocess engineering, the exploitation of interaction between biocatalysts and solid surfaces has made it possible to develop new biocatalyst applications such as biosensors (Pinheiro et al., 1987). Bioseparation and purification of proteins by chromatography and foam fractionation involve competitive adsorption of different proteins at solid-liquid and gas-liquid interfaces (Anand et al., 1995). Biocompatibility of clinical implants, mammalian and bacterial cell adhesion to surfaces are also heavily influenced by the presence of proteins (Horbett et al., 1982). Furthermore, practical problems such as contact lens fouling, foaming of protein solutions, and fouling of equipment in the food industry, are direct consequences of the relatively high surface activity of proteins (Andrade et al., 1985). The behavior of enzymes and antibodies at interfaces is not only important in a range of applications, but it also provides a rather direct route to understand proteins at interfaces , because these

protein molecules have natural properties (active site, ligand binding site) that may be readily probed to determine the functional consequences of adsorption (Horbett et al., 1987).

In their application to various biological, medical, and food systems, enzymes are usually immobilized on a solid surface (Pinheiro et al., 1987). One of the main problems, however, as a consequence of enzyme immobilization is a significant reduction in catalytic activity. It has been generally recognized that proteins undergo conformational changes upon adsorption to solid surfaces (McGuire et al., 1995; Billsten et al., 1995), hence conformational change is thought to be one of the main reasons for activity reductions in immobilized enzymes. There is limited information concerning how the thermostability of an enzyme affects its activity at a solid-liquid interface, and research concerning the structural and functional consequences of protein adsorption to surfaces is still in an early stage of development. This information is important not only for the theoretical aspects but also for the practical aspects such as preparation of immobilized enzymes with high activities (Kondo et al., 1992). Structural stability has already been directly related to conformational changes upon adsorption of bacteriophage T4 lysozyme (Billsten et al., 1995). Differences in enzymatic activity measured by T4 lysozyme mutants ought to be explainable in the same terms.

In this study, we investigated the effects of different thermostabilities of T4 lysozyme mutants on the extent of activity reductions upon adsorption to 9 nm silica particles in a 1 : 1 molecular ratio. Lysozyme catalyzes the hydrolysis of the $\beta-1, 4$ glycosidic linkages between the alternating units of N - acetylmuramic acid and N - acetylglucosammic in bacterial peptidoglycan (Hardy et al., 1991), thus providing a basic

method to measure the lysozyme activity. Spectrophotometry is a classical method of activity measurement based on the continuous reading of the change in turbidity of a bacterial suspension. This is a relatively simple and rapid technique for the measurement of lysozyme activity with high sensitivity and good accuracy (Shugar 1952). Agar plate bioassays have been improved in sensitivity (Gosnell et al., 1975) and are intuitive and easy to perform.

The objective of this study was to provide direct information on the relationship between stability ($\Delta\Delta G$) of a T4 lysozyme variant and its subsequent activity in an adsorbed state. The conformation of adsorbed molecules may not be identical to their solution conformation, since interfacial adsorption may cause denaturation and partial unfolding of the adsorbed proteins. Changes in enzymatic activity may also reflect the protein's conformational change upon adsorption. By comparing the different thermostabilities of T4 lysozyme variants with their different enzymatic activities, we can better understand how structural changes affect protein function.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein adsorption at solid surfaces

Protein adsorption onto various interfaces has been the subject of many studies. The behavior of protein adsorption at solid surfaces is mainly affected by three factors: protein characteristics, solution characteristics and solid surface characteristics. Horbett and Brash (1987) summarized the molecular properties of proteins that may influence their adsorption to solid surfaces, including size, isoelectric point, charge distribution, structure and stability in solution, and placement and nature of hydrophobicity. They pointed out that proteins near their isoelectric point may adsorb to surfaces more easily. Less stable and more hydrophobic proteins may also favor adsorption. In a previous study, Podhipleux (Podhipleux et al., 1994) investigated the effects of charge distribution of bacteriophage T4 lysozyme mutants adsorbing at a silanized silica surface. By using two different models, they found that proteins adsorbed at the interface more tightly and occupied a greater interfacial area with amino acid substitution of Lysine than with glutamic acid. They attributed these effects to the location of the substitution rather than the protein's net charge. McGuire et al. (1995) investigated the influence of bacteriophage T4 lysozyme at both hydrophobic and hydrophilic silica surfaces, and found no simple relationship between protein net charges and surface behavior, suggesting that the location of the charge replacements had its major effect on surface behavior. The

environmental conditions such as pH, temperature, ionic strength of solution, solute type and concentrations along with the motion of fluid also play an important role in protein adsorption. Norde and Lyklema (1978) suggested that the degree to which pH affects the adsorption of a protein is determined by its conformational stability. Kondo and Higashitani (1992) explained the pH dependence of the plateau adsorption of large proteins in terms of lateral interaction, and found that the plateau adsorption at a negatively charged surface significantly decreases with increasing pH. Tsugita et al. (1968) described the effects of temperature and pH on the enzymatic activity of bacteriophage T4 lysozyme variants. They found the optimum pH to be around 7.4, with enzyme inactivation observed at temperatures above 40°C. The hydrophobicity and the hydrophilicity of a solid surface probably are the major factors affecting protein adsorption at solid surfaces and are also well studied. Ho et al. (1995) investigated the adsorption of human low-density lipoprotein (LDL) onto a silica-octadecyldimethylsilyl (C18) gradient surface by performing two identical TIRF adsorption experiments with protein-labeled LDL and with lipid-labeled LDL. Two different kinds of surfaces were used, a transport-limited negatively charged silica surface, and a hydrophobic C18 silica gradient surface. Their results showed that the apparent affinity among LDL molecules for each other in the bulk phase decreases with increasing surface hydrophobicity, and they confirmed that both protein and lipid components of LDL became adsorbed on the silica-C18 gradient surface. Malmsten (1995) also studied the hydrophobic interaction between the protein and the interface, and found that the more hydrophobic the surface, the more adsorption will occur at the interface. Other characteristics of solid surfaces such as the energetic heterogeneity and the surface area also affect protein adsorption. Nanoparticles can play an important

role in adsorption of enzymes due to the large specific surface area and high surface free energy (Chen et al.,1996).

The characteristics described above are only some of the factors that govern protein adsorption. There are still many aspects not yet clear, such as the conformational rearrangement of proteins upon adsorption.

2.2 Protein stability and conformational changes

The ordered, functional structures of proteins reflect two tendencies that are often opposed. On one hand, proteins fold to minimize their free energy. On the other hand, they organize themselves to recognize a ligand or a transition state. In an aqueous environment, minimizing free energy leads to well-packed hydrophobic interiors and hydrophilic exteriors. Maximizing enzyme function leads to active-site clefts where charged and polar groups may be sequestered from water, and hydrophobic patches may be exposed to solvent (Shoichet et al.,1995). It is well known that folded proteins are only marginally stable. Many attempts have been made to increase the thermal stability of proteins. Most of them have focused on the introduction of non-native disulfide cross-links by site-directed mutagenesis (Matthews, 1987). Villafranca et al. (1987) found that an engineered disulfide bond in dihydrofolate reductase increases stability with respect to unfolding with Guanidine hydrochloride but decreases stability toward thermal denaturation. Matthews et al. (1987) found another approach for protein stabilization by utilizing amino acid substitutions which decrease the configurational entropy of unfolding

of the polypeptide backbone. Matsumura et al. (1988) studied hydrophobic effects on protein stability by replacing the isoleucine at amino acid position three of bacteriophage T4 lysozyme. They concluded that changes in the thermal stability of the protein are directly related to the hydrophobicity of the substituted residue with the hydrophobic stabilization being proportional to the reduction of the surface area accessible to the solvent on folding.

Thermal stability is one of the important properties of protein adsorption at an interface. McGuire et al. (1995) investigated structural stability effects on the adsorption and dodecyltrimethylammonium bromide (DTAB)-mediated elutability of bacteriophage T4 lysozyme mutants at silica surfaces with *in situ* ellipsometry. They found the DTAB-mediated elutability of each variant at each surface increased with increasing protein stability. Billsten et al. (1995) studied the structural changes of T4 lysozyme upon adsorption to silica nanoparticles by using circular dichroism (CD), and found that about 12% of the α–helix of Wild Type was lost. For the higher stability mutant cysteine, about 9% of the α–helix was lost, and for the lower stability mutant tryptophan, about 29% of the α–helix was lost. A similar study by Tian (1996, thesis) confirmed that the more unstable the protein, the greater the rate and extent of secondary structure loss upon adsorption.

In general, thermostability is an important property of a protein at an interface, suggesting an intrinsic relationship between structural stability and confomational changes upon adsorption.

2.3 Characteristics of T4 lysozyme and its enzymatic activity

Bacteriophage T4 lysozyme is an enzyme produced by inserting a lysozyme

expression vector from a T4 bacteriophage into a host cell such as *Escherichia coli (E.*

coli). It is a hydrolytic enzyme that cleaves glycosidic bonds in the cell walls of

susceptible bacteria and leads to cell lysis (Grütter et al., 1982). T4 Lysozyme is a small

and well characterized protein with 164 amino acid residues. It has a molecular weight of

approximately 18,700 (Matthews et al., 1974) and a size of about 54×28×24Å (Weaver et

al., 1987). T4 lysozyme is a basic molecule with an isoelectric point around pH 9, and an

excess of nine positive charges at neutral pH (Sun et al., 1991). T4 lysozyme contains

two distinct domains. Residues 1 - 60 form the N-terminal domain which contains two α-

helices and all of the β-sheets. Residues 80 - 164 form the C-terminal domain which

contains 7 α-helices and no β-sheets. The two domains are joined by a long α-helix (

from 60 - 80) that traverses the length of the molecule. Residues 159 - 164 appear to

form a distorted α-helix (Tom et al., 1987). Overall, the wild type T4 lysozyme molecule

contains approximately 60% α-helix of its total secondary structure (Toumadje et al.,

1992). A diagram of the α-carbon backbone of T4 lysozyme is illustrated in Figure 2.1.

Bacteriophage T4 lysozyme is often chosen as the model protein for research

because it is extremely well characterized. The three dimensional structure and surface

morphology are known and numerous variants of this protein have been produced through

site-directed mutagenesis and characterized with respect to their deviation on crystal

structure and thermodynamic stability from the wild type protein. Isoleucine at

position 3 (Ile 3), located as shown in Figure 2.1, has been replaced with 13 different amino acid residues by site-directed mutagenesis. It was shown that hydrophobicity of the residue at position 3 influences the stability of the whole molecule, since Ile 3 contributes to the major hydrophobic core of the C-terminal lobe and helps to link the N- and C-terminal domains. The side chain of Ile 3 contacts the side chain of methionine at position 6, leucine at position 7, cysteine at position 97 and isoleucine at position 100 (Matsumura et al., 1988). The differences in structural stability between wild type and mutants are given by $\Delta\Delta G$, which indicates the difference between the free energy of unfolding of the mutant protein and that of the wild type at the melting temperature of the wild type lysozymye. The thermal stabilities of the mutants relative to wild type T4 lysozyme are - 2.8 Kcal/mol and 1.2 Kcal/mol for tryptophan and cysteine mutants respectively (Matsumura et al., 1988). The substitution of Ile 3 with cysteine resulted in a more stable mutant, because a disulfide bond forms between Cys 3 and Cys 97, which leads to an increased stability for this mutant (Tom et al., 1987). The replacement of Ile 3 with tryptophan resulted in a less stable mutant, because the substitution caused unfavorable steric interactions and resulted in unsatisfied hydrogen bonds. The substitution at position 3, which is near the amino terminus, can be made without major changes to protein structure in solution (Matsumura et al., 1988).

Matthews et al. (1974) described the three dimensional structure of T4 lysozyme as a polypeptide backbone which folds into two distinct lobes linked in part by a long helix. In the region between the two lobes, there is a cleft which deepens into a hole or cavity, about 6 - 8 Å in diameter, extending from one side of the molecule to the other. This opening is closed off by side chains which extend to within 3 - 5 Å of each other. A
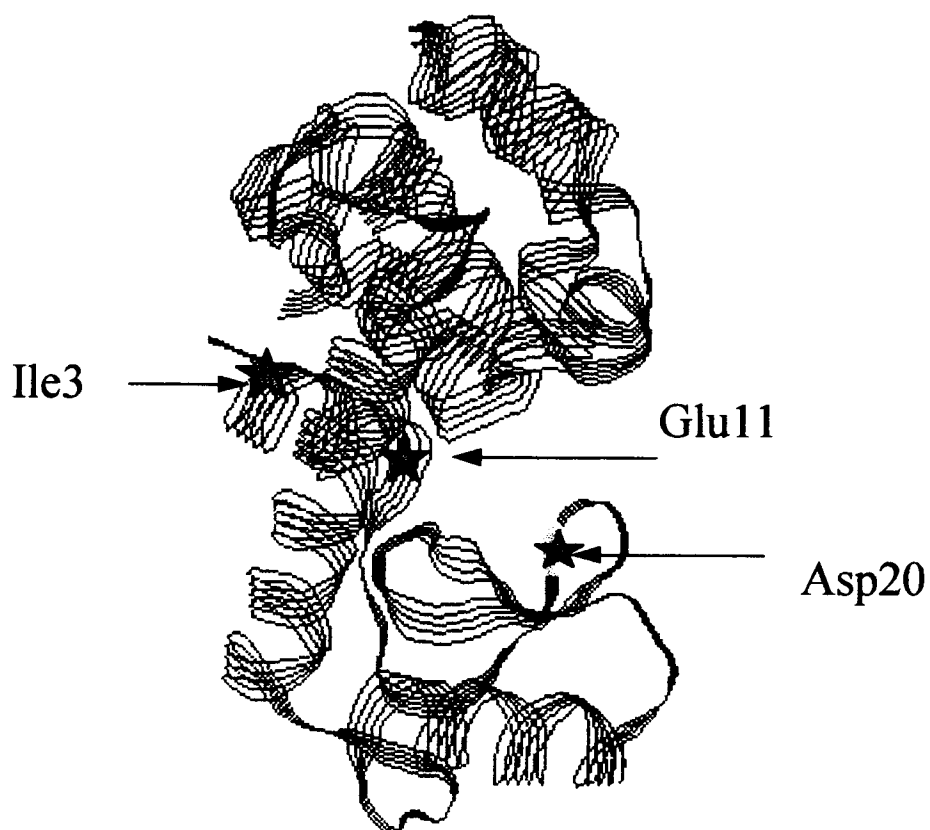
Fig. 2.1 A threads and ribbons model of the α-carbon backbone of bacteriophage T4 lysozyme, showing the location of the mutation Ile 3→Trp and Ile 3→Cys. The active-site residues Glu 11 and Asp 20 are also shown.

number of mutant lysozymes in which residues in the vicinity of the opening are modified have markedly reduced catalytic activity, suggesting that this region of the molecule may be catalytically important. The active site was located at residues Glu 11 and Asp 20 (Hardy et al. 1991, Matthews et al. 1974), and Masazumi et al.(1989) found by building an engineered disulfide bond into the active-side cleft, the activity of T4 lysozyme could be switched on or off. Grütter et al. (1982) studied the effect of single amino acid substitutions on the structure, function and stability of T4 lysozyme. They found that a mutant lysozyme for which the location of the amino acid substitution was on the far side of the molecule (25 Å from the active site) had its catalytic activity redued to 4% that of the Wild type enzyme. According to De Roos et al. (1996), the activity of enzymes at interfaces has been studied in three ways. First, the substrate is at the interface and the enzyme is in solution. In these cases, full or diminished enzyme activities were found. Second, enzyme adsorbed onto an air/water interface are transferred by Langmuir - Blodgett - like techniques and resuspended afterwards. Mostly, little or no activity was observed. Third, the interface holds the enzyme (activity *in situ*) and the substrate is in the bulk phase. This has mainly been done at the solid-liquid interface and at the air-water interface. Mostly partial to complete inactivation was observed. Kondo et al. (1995) investigated the temperature dependence of activity and conformational changes in α-Amylases with different thermostabilities upon adsorption to ultrafine silica particles. They found that the relative activity after adsorption was strongly affected by the temperature, with increasing temperatures from 4 to 40°C resulting in decreasing activities. The extent of activity reduction upon adsorption was larger when lower amounts of protein were present.

There are many excellent, well understood methods available for measuring the quantity of protein adsorbed at an interface, but few good techniques exist for determining qualitative features such as the structure or orientation of an adsorbed protein, or for studying the relationship of a particular aspect of the adsorption process to its influence on other process at the interface (e.g., cellular interactions). Thus a rather large amount of information about the behavior of protein at interfaces must still be obtained by further research.

CHAPER 3

MATERIALS AND METHODS

3.1 Production and purification of T4 lysozyme

T4 lysozyme wild type, two stability mutants and an inactive mutant were produced from transformed cultures of *E. coli* RR1 which were obtained from the lab of Professor Brian Matthews (Institute of Molecular Biology, Eugene, OR). These synthetic mutants of T4 lysozyme were produced by replacing isoleucine with cysteine or tryptophan at position 3 to produce the stability mutant Ile $3 \rightarrow$ Cys and Ile $3 \rightarrow$ Trp, and by replacing glutamic acid at position 11 with Alanine to produce the Glu $11 \rightarrow$ Ala inactive mutant. Expression and purification of T4 lysozyme and its mutants were performed following established procedures (Tom et al., 1987).

Stock cultures of E.coli RR1 which carry an ampicillin resistant gene were originally stored at -80°C. Cells bearing the desired mutant lysozyme expression vector were aseptically transferred to 200ml LB - H broth (1g tryptone, 0.5g yeast extract, 0.5g NaCl, 0.1ml 1N NaOH and 200ml distilled deionized water DDW) with 10mg ampicillin. The cells were allowed to grow at 37°C with agitation for 6 - 8 hours before being transferred to 4.8 liters of sterilized LB broth (57.6g tryptone, 24g yeast extract, 48g NaCl, 4.8g glucose and 4.8 liter DDW) with 0.4g ampicillin and 1.5ml tributyl phosphate (Sigma Chemical Co., St. Louis, MO). A 7 - liter autoclaved fermenter was used. The

culture was grown at 35°C by using a water bath with a circulating system (Model 1120, VWR Scientific, Portland, OR). Agitation was maintained at 600 rpm with a speed controller (ADI 1012, Applikon Dependable Instruments, Schiedam, Holland) while the air flow rate was maintained at 0.8kg/s. The culture grew under these conditions until its optical density at 595nm (DU 62 Spectrophotometer, Beckman Instruments, Inc. Futterlon, CA) was between 0.8 and 1.0 (about 2 - 3 hours). Then the temperature of the culture was lowered to 30°C, and the expression of the gene which codes for lysozyme production was induced with the addition of 750mg isopropyl-β-thiogalactoside (Sigma Chemical Co., St. Louis, MO) dissolved in 10ml DDW. The fermentation was allowed to continue for another 110 minutes at 30°C with an air flow rate of 0.52kg/s and an agitation of 200rpm. The lysozyme-rich broth was then harvested and centrifuged.

The first round of centrifugation was done at 4°C, 13k rpm (JA-14 Rotor, Beckman Model J2-MI centrifuge, Beckman Instruments Inc., Palo Alto, CA) for 25 minutes. The mutant protein existed in both the supernatant and pellet fraction after the first spin. The supernatant was re-centrifuged at 13k rpm for 40 minutes. After this second spin, the supernatant was ready for dialysis, and the second spin pellets were discarded. The first spin pellets were combined and resuspended with 20ml of 10mM, pH 7.4 Tris buffer and 180ml of pH 6.6 lysis buffer (0.1M sodium phosphate buffer, 0.2M NaCl, 10mM $MgCl_2$). An addition of 2ml of 0.5M ethylenediamine tetraacetic acid, pH 8.0 (Sigma Chemical Co.) was added to the resuspended pellet. The mixture was then stirred at 4°C for about 12 hours. Then 0.02mg of deoxyribonuclease I (Dnase I, crude powder from bovine pancreas, Sigma Chemical Co.) and 2ml of 1M $MgCl_2$ were added to the pellets. This suspension was stirred at room temperature for 2 hours, and then

centrifuged at 20k rpm (JA-20 Rotor, Beckman Model J2- MI Centrifuge, Beckman Instruments Inc., Palo Alto, CA) for 30 minutes. The pellets of the last centrifugation were discarded and the supernatant was dialyzed.

Each 1100ml of combined supernatant was dialyzed in a 1200ml plastic fleaker against about 4 liters of DDW with Spectra/ Por regenerated cellulose hollow fiber bundles (MWCO18,000, Spectrum Medical Industries, Inc., Houston, TX) until the conductivity (Cond/TDS, Corning Glass Works, Corning, NY) of the supernatant was less than 2 µmho/cm. The whole dialysis process required approximately 48 hours.

The dialyzed solution was then loaded onto a CM Sepharose ion exchange column (CM Sepharose CL - 6B CCL - 100, Sigma Chemical Co.) which was previously equilibrated with 50mM Tris buffer at pH 7.25. As the dialysis solution moved through the column, a thick white band of protein forming on top of the Sepharose could be observed. After all the solution has passed through the column, the column was then rinsed with 150 - 200ml of 50mM Tris buffer to remove any yellow colored materials that would interfere with the UV signal during fraction elution of the lysozyme. A salt gradient of 50mM to 0.3M NaCl in 50mM Tris buffer was used to elute the protein from the column into a fraction collector (Frac -100, Pharmacia LKB Biotechnology, Alameda, CA). The absorbance (at 280nm) of the eluant corresponding to each fraction was recorded on a chart recorder. The fractions containing protein were collected and transferred into a Spectra/Por molecular porous membrane tubing (MWCO 12 - 14K, Spectrum Medical Industries, Inc.) for 12 hours of dialysis against 50mM, pH 5.8 sodium phosphate dialysis buffer (25.09g NaH_2PO_4, 2.58g Na_2HPO_4, 0.8g NaN_3, 4L DDW) with a volume of 20 times the eluant volume. These procedures were all performed at 4°C.

The protein solution from the dialysis tubing was loaded onto a SP Sephadex column (SP Sephadex C50, Sigma Chemical CO.) for concentration. The lysozyme was eluted from the column with 0.55M, pH 6.5 sodium phosphate elution buffer (4.595g NaH_2PO_4, 2.371g Na_2HPO_4, 0.1g NaN_3, 16.071g NaCl, 500ml DDW) and the concentrated solution was stored in 1.5ml vials at 4°C without further treatment. The concentration of lysozyme in each vial was determined according to Beers law by measuring the absorbance at 280nm with a UV spectrophotometer. Samples were diluted 1 : 100 with 0.55M sodium phosphate elution buffer at pH 6.5, and the absorbance was divided by the molar absorption coefficient of 1.28 for Wild type and all variants except the Tryptophan mutant, which was divided by 1.46.

3.2 Isolation of peptidoglycan from *Escherichia. coli* cells

Isolation of peptidoglycan from *E. coli* was based on the procedure of Becktel and Baase (1985) with some modifications. *E. coli* cells were purchased from Sigma Chemical CO. (Lyophilized cells of Strain B, ATCC 11303). 50g of *E.coli* cells were stirred and suspended in 250ml of 0.9% NaCl solution. The suspension was pressed at 16,000 - 18,000 psi using a French Pressure Cell Press (SLM AMINCO, SLM Instruments Inc.) with a cell size of 40ml. The resulting slurry was then centrifuged at 14.5k rpm (JA-20 Rotor, Beckman Model J2- MI Centrifuge, Beckman Instruments Inc., Palo Alto, CA) for 1.5 hours. The supernatant was discarded and the resulting pellet was resuspended in 200ml of 1M NaCl solution and heated to 100°C for 45 minutes. The suspension was re-

centrifuged at 5k rpm for 20 minutes. The supernatant was discarded and the pellet was washed by stirring with 1M NaCl solution. This procedure was repeated three times and then the pellet was resuspended in 65ml DDW to produce a total volume between 100 - 200ml. The optical density (OD) of a 1 : 100 dilution at 600nm was approximately 1.0. This cell wall slurry was then slowly added to a boiling 300ml solution of 10% SDS (Sodium Dodecylsulfate, L - 3771, Sigma Chemical CO.) and boiled for an additonal 20 minutes. After cooling to room temperature, the cell wall solution was centrifuged again at 20k rpm, 25°C for 1.5 hours. The supernatant was discarded and the pellet was resuspended in 250ml DDW. This centrifugation/washing procedure was repeated 5 times. The last two times, the pellet was resuspended with 50mM, pH 7.4 phosphate buffer. The final volume of cell walls was about 225ml, and the OD was about 1.3 at 600nm.

3.3 Nanoparticles

Colloidal silica particles (food grade quality) were provided by EKA - Nobel Inc. (Marietta, GA). The original stock solution contained 7.8×10^{17} particles/ml. The particles were 9 nm in diameter and were used without further modification. Nanoparticles were used in this study as the solid surfaces for protein adsorption. For T4 lysozyme with a molecular weight of 18,700, 0.1mg/ml of the lysozyme solution contained 3.2×10^{15} particles/ml, which needed 4.1μl of nanoparticles per milliliter of lysozyme solution to make a protein to particle ratio of 1 : 1.

3.4 Measurement of T4 lysozyme activity using spectrophotometric techniques

3.4.1 *Escherichia. coli* cell walls as the substrate

Measurement of T4 lysozyme activity was performed using a spectrophotometric technique based on the procedure of Shugar (1952) and Tsugita et al. (1968) with some modifications. Stock *E. coli* cell wall solution was diluted with 50mM, pH 7.4 phosphate buffer to give an OD around 0.6 - 0.7. The diluted *E. coli* cell wall substrate, along with the substrate-nanoparticle solution, the substrate - T4 lysozyme solution, and the substrate - T4 lysozyme-nanoparticle complex (2ml substrate, 0.1ml sample) were individually scanned from 270nm - 800nm using a Scanning Spectrophotometer (UV - 2100 PC Shimadzu, UV - VIS/UV - VIS - NIR Scanning Spectrophotometer)within 2-3 minutes. The wavelength of 450nm was chosen for the assays.

Stock WT solution was diluted with the same buffer to give sample concentrations ranging from 0.1mg/ml to 1.0mg/ml, which resulted in a reaction concentration (the concentration of T4 lysozyme in the substrate mixture) ranging from 4.8μg/ml to 50μg/ml for a reaction of 2ml substrate and 0.1ml sample. These samples were then tested for activity (2ml substrate, 0.1ml sample) to determine the linear region of this assay.

WT and Ile 3→Trp were diluted with the same buffer to give sample concentrations ranging from 0.1mg/ml to 0.7mg/ml, which resulted in reaction concentrations ranging from 4.8 μg/ml to 33 μg/ml. The diluted samples were then separated into two parts, one part as free T4 lysozyme, another part as adsorbed T4 lysozyme in which the amount of nanoparticles resulting in a 1 : 1 protein to particle ratio was added. The protein-nanoparticle complexes were then allowed to adsorb for 90

minutes before the assays were performed. For each assay, 0.1ml of T4 lysozyme or lysozyme-nanoparticle complex was added into 2ml *E. coli* cell wall substrate, quickly mixed well and scanned immediately at 450nm for 1 minute. For each concentration of T4 lysozyme, four replicate data were taken. The assays were carried out at room temperature.

3.4.2 *Micrococcus Lysodeikticus* cell walls as the substrate

Micrococcus lysodeikticus cells (*M. lysodeikticus* cells) were purchased from Sigma Chemical CO.(ATCC No. 4698, M - 3770). 0.15g of *M. lysodeikticus* cells were dissolved in 1 liter 50mM, pH 7.4 phosphate buffer, and stirred at least 4 hours at 4°C before use to allow the cells to completely diffuse. The OD of the cell suspension was adjusted to be between 0.6 - 0.7. The *M. lysodeikticus* cell substrate along with the substrate-T4 lysozyme, the substrate-T4 lysozyme-nanoparticle complex were put into the scanning spectrophotometer individually for spectral scanning. The wavelength of 450nm again was chosen for the assays. Stock WT solution was diluted with the same buffer to give sample concentrations ranging from 0.09mg/ml to 1.3mg/ml, which resulted in a reaction concentrations ranging from 4μg/ml to 62μg/ml. These samples were tested for the linear region of concentrations using *M. lysodeikticus* cell suspension as substrate.

Stock WT, Ile 3→Cys, Ile 3→Trp and Glu 11→Ala were diluted with the same buffer to give sample concentrations ranging from 0.4mg/ml to 1.4mg/ml, which resulted in reaction concentration ranging from 19μg/ml - 69μg/ml. Samples were again separated into two parts for the free and adsorbed T4 lysozymes (1 : 1 ratio). Assays used *M.*

lysodeikticus cell wall as the substrate and were performed in a manner similar to the assays using *E. coli* cell wall substrate.

3.5 Measurement of T4 lysozyme activity using agar plate bioassays

Measurement of T4 lysozyme activity using an agar plate bioassay was based on the procedure of Gosnell et al. (1975). Bacto - agar (DIFCO Laboratories, Detroit Michigan USA) was dissolved in DDW (7.5g per 500ml) and 0.1g of NaN_3 was added to avoid contamination. 1.5g of *M. lysodeikticus* cells (ATCC No. 4698, M - 3770, Sigma Chemical CO.) was added to give a seeding concentration of 0.3g/100ml. The mixture was stirred and heated until it was uniform, then autoclaved for 15 minutes (121°C). 50ml of this media was poured into each sterile plastic petri dish (Falcon 1058, 150 × 15 mm). The agar plates were then cooled down to allow solidification and were stored at 4°C until used. The large end of a sterile Pasteur pipet with a diameter of 6mm was used to punch wells in the agar gel, and the small tip of the pipet was used to remove the plugs. Each well could hold up to 80μl of sample. Stock WT, Ile 3 → Trp and Ile 3 → Cys solutions were diluted with 0.55M, pH 6.5 sodium phosphate elution buffer to give concentrations of 0.5mg/ml. Samples were separated into two parts, and the amount of nanoparticles which gave a 1 : 1 protein to particle ratio was added to one part. The protein-nanoparticle complexes were allowed to adsorb for 90 minutes. Each well received a 50μl aliquot of free enzyme, enzyme - nanoparticle complex, or buffer and nanoparticle control. The plates were then incubated at 35°C and the diameters of the clear zones were

measured after 24 hours. Different concentrations ranged from 0.01 to 0.52mg/ml of WT, Ile 3→Trp, Ile 3→Cys solutions and their nanoparticle complexes were also assayed in duplicate. The $\log_{10}$ concenrtation of each lysozyme control (unit/ml) was plotted against the square of the corresponding zone width to obtain a line for comparing the lysozyme activity of the samples.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Determining the best wavelength for absorbance measurements

The peptidoglycan substrate, T4 lysozyme and nanoparticles were each scanned from 270nm to 800nm using both *E. coli* cell wall and *M. lysodeikticus* cell wall as substrates within 2-3 minutes. Representative graphs are shown in Figure 4.1a and 4.1b. The results showed that there were no unexpected peaks between 400nm and 800nm for any of the lysozyme variants or their nanoparticle complexes. The wavelength of 450nm was chosen for the assay because it gave a relatively higher absorbance than that of the wavelength above 450nm, thus requiring a less concentrated substrate for the assay. This is consistent with previous spectrophotometric assays for lysozyme by Tomizawa et al. (1995) and Shugar (1952).

4.2 Determining the linearity of the activity assay

WT reaction concentrations ranging from 4.8µg/ml to 50µg/ml (using *E. coli* cell walls as a substrate) and ranging from 4µg/ml to 62µg/ml (using *M. lysodeikticus* cell
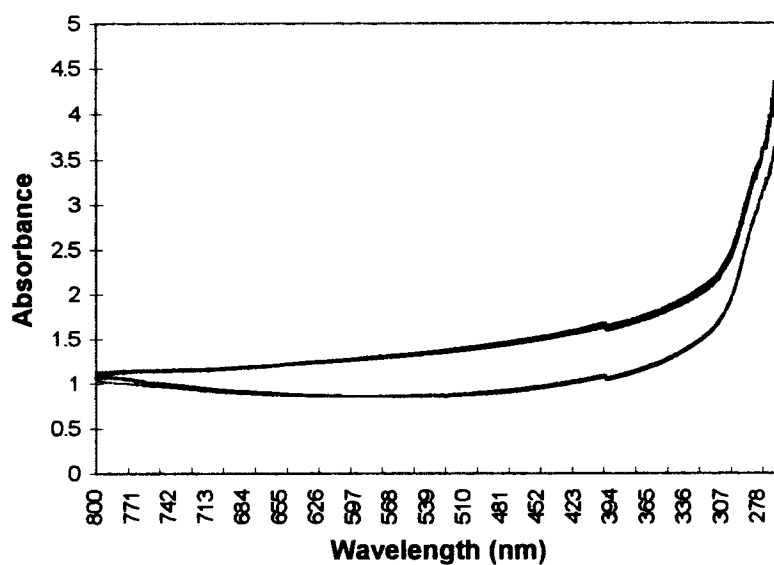
Figure 4.1a. The scanning spectra of *E. coli* cell wall substrate with WT and nanoparticles. Upper lines are *E .coli* cell wall substrate and the substrate with nanoparticles; lower lines are substrate with WT and substrate with WT-nanoparticle complexes.
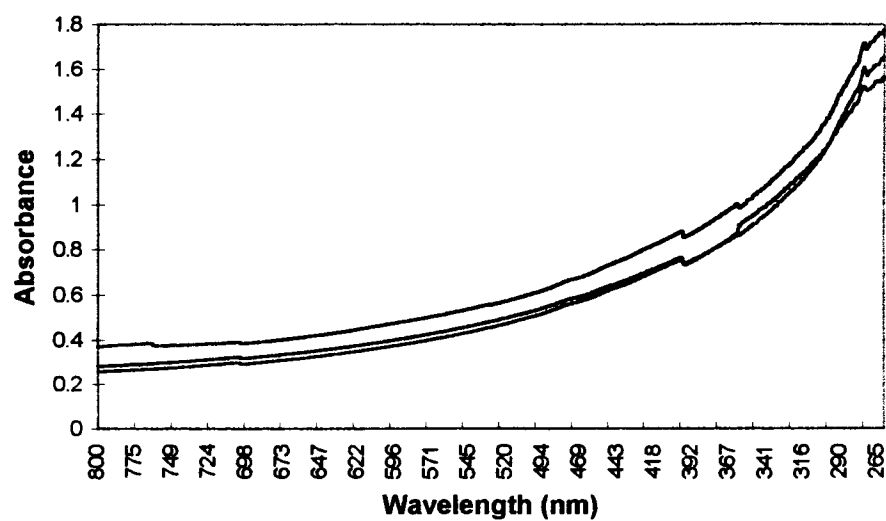
Figure 4.1b. The scanning spectra of *M. lysodeikticus* cell wall substrate (upper line), substrate with WT (lower line) and substrate with WT-nanoparticle complexes (middle line).

walls as a substrate) were tested to determine the linearity of the activity assay. The linear

region of the curve appeared to be between 4.8μg/ml to 37μg/ml for *E. coli* cell walls,

while the whole testing region was linear for *M. lysodeikticus* cell walls (Figure 4.2a,

4.2b). Similar results were obtained for the Ile 3→Cys and Ile 3→Trp mutants. They

were similar with the concentration scale used by Tsugita (1968) and Shugar(1952).
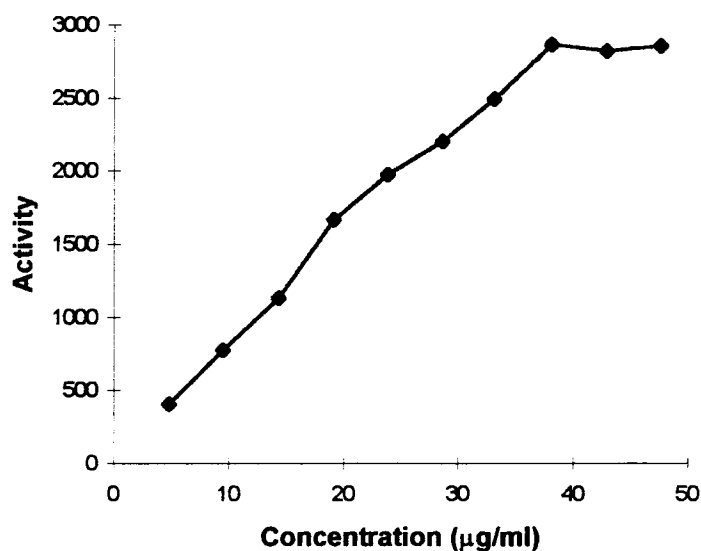

Figure 4.2 a The linear region of the dependence of activity on concentration for WT
solution using *E. coli* cell walls as substrate. Activity = $mA_{450} \times 10^4$ /min.
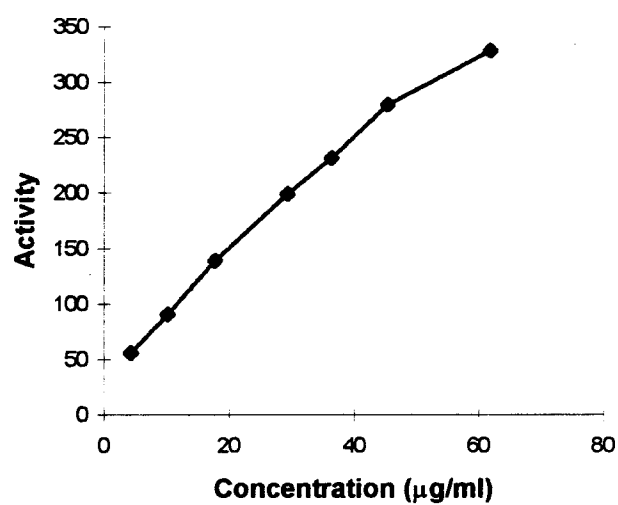
Figure 4.2 b The linear region of the dependence of activity on concentration for WT solution using *M. lysodeikticus* cell walls as substrate. Activity = $mA_{450} \times 10^4$ /min.

4.3 Verifying the stabilities of protein, particle and their substrate complex in solution

The inactive lysozyme mutant (Glu 11→Ala) was tested both before and after adsorption to nanoparticles using spectrophotometric techniques with *M. lysodeikticus* cell walls as the substrate. No activity was found. The reaction kinetics of Glu 11→Ala along with substrate control are shown in Figure 4.3. From the results obtained with the inactive mutant Glu 11→Ala, we eliminated the possibility that the protein-particle complexes simply bound to the substrate and precipitated without reflecting true enzymatic activity. By including an inactive mutant along with active T4 lysozymes, it was possible to demonstrate that the curves obtained in the spectrophotometric assays were related to the enzymatic activities of T4 lysozymes, and were not simply artifacts associated with the lysozyme-particle-substrate system.

4.4 Evaluation of T4 lysozyme activity with and without nanoparticles using *E. coli* cell walls

Wild type (WT) and Ile 3→Trp were tested before and after adsorption to nanoparticles at a 1 : 1 protein to particle ratio, after 90 minutes adsorption. Representative reaction kinetics graphs of WT and Ile 3→Trp are shown in figure 4.4a. The standard curves of enzymatic activity were plotted in Figure 4.4b. For both WT and Ile 3→Trp, T4 lysozyme demonstrated less activity when they adsorbed to nanoparticles. WT always displayed more activity than did Ile 3→Trp, regardless of whether
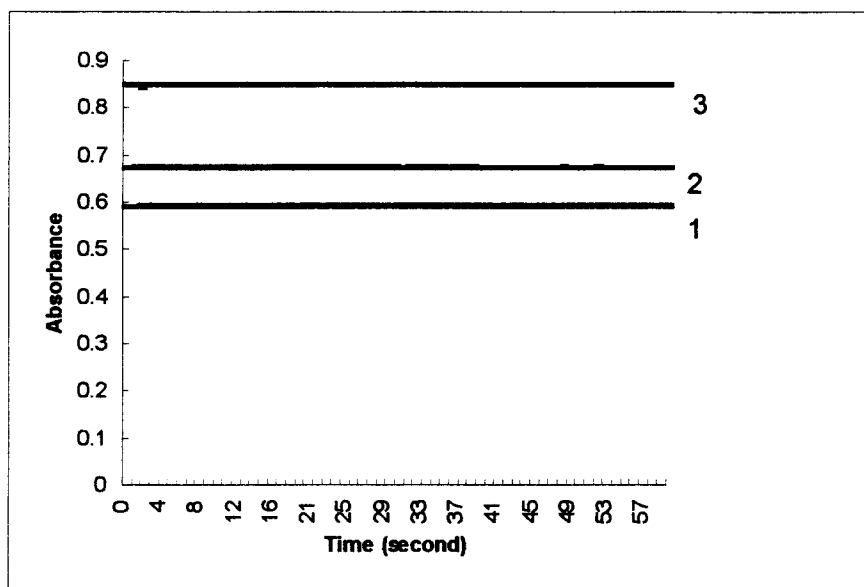
Figure 4.3 The reaction kinetics of Glu 11→Ala, Glu 11→Ala with nanoparticle complexes, and *M. lysodeikticus* cell wall substrate control. Line 1: substrate, line 2: Glu 11→Ala and substrate, line 3: Glu 11→Ala with nanoparticle and substrate.
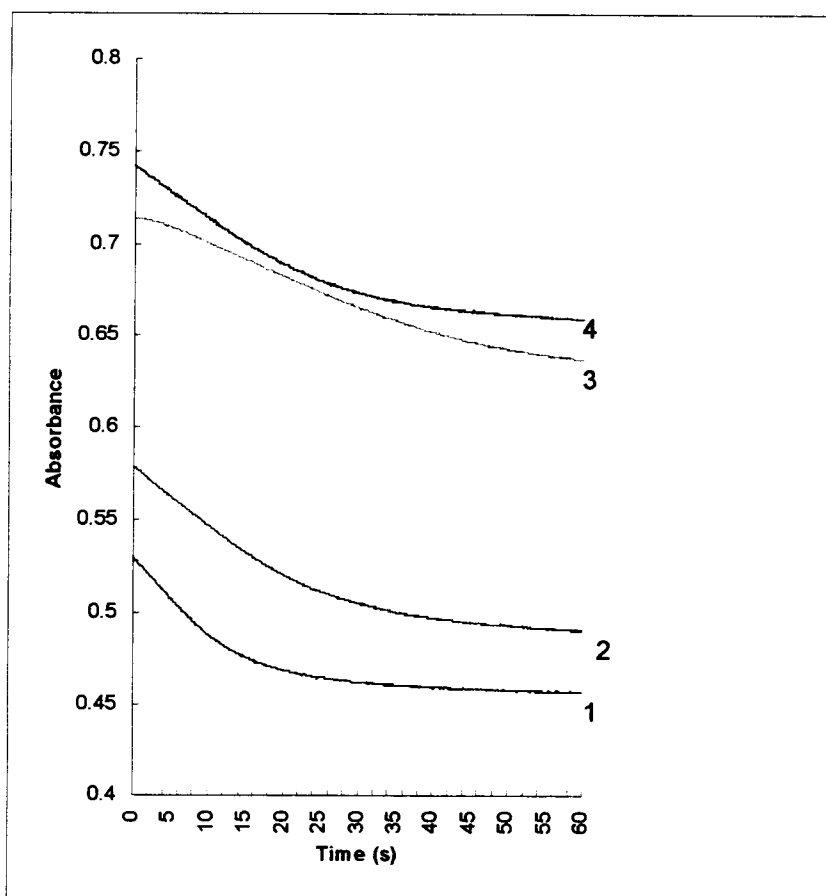
Figure 4.4a The reaction kinetics of WT and Ile 3→Trp with and without nanoparticles using *E. coli* cell walls. Line 1: WT (24µg/ml), line 2: Ile 3→Trp (24µg/ml), line 3: Ile 3→Trp (24µg/ml) with NP (nanoparticles), line 4: WT (24µg/ml)-NP.
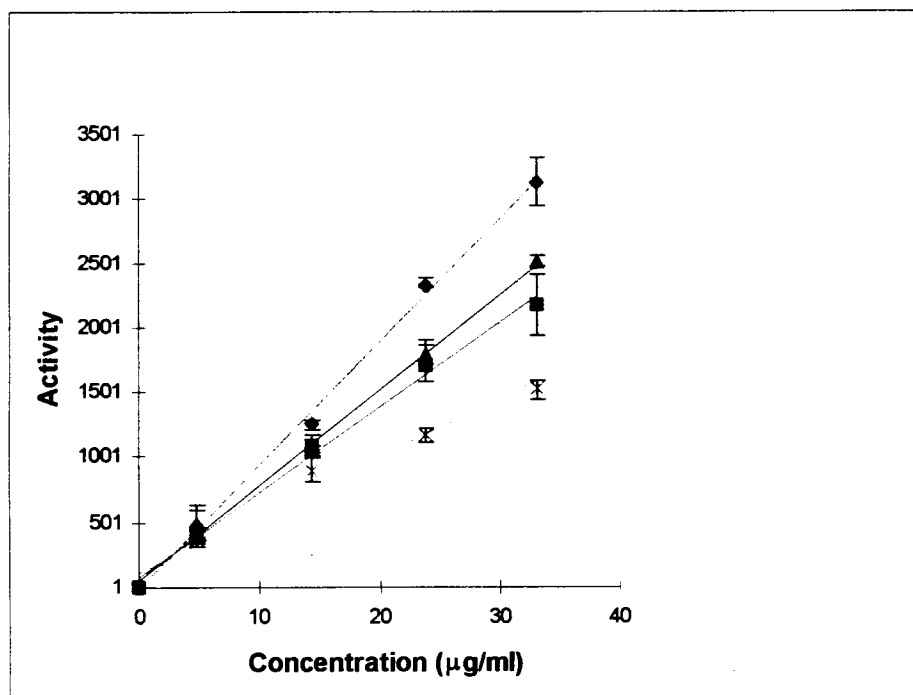
Figure 4.4b The standard curves of WT, Ile 3→Trp and their nanoparticle complexes using *E. coli* cell walls as substrate. ◆,WT; ▲, WT-nanoparticle complexes; ■, Ile 3→Trp; ×, Ile 3→Trp with nanoparticle complexes. Activity = $mA_{450} \times 10^4/min$.

nanoparticles were present. Additionally, Ile 3→Trp lost more activity than that of WT when absorbed to nanoparticles. The enzymatic activity of free and adsorbed T4 lysozymes using *E.coli* cell walls as substrate are also shown in Table 4.4. The activity of T4 lysozyme was calculated according to the following definition: <u>one unit of activity is the amount of enzyme needed to decrease A_{450} of a peptidoglycan by one absorbance per minute multiplied by a factor of 10^4, that is $\Delta mA_{450} \times 10^4/min.*mg$</u> (Knight et al., 1987).

From Table 4.4, it is apparent that both WT and Trp lost part of their activity after adsorption to nanoparticles. There is the possibility that the active-site of lysozyme is obstructed by adsorption onto the interface resulting in steric hindrance and a decreased capability of the enzyme to react with the substrate. Furthermore, the adsorption of a lysozyme molecule onto the solid surface may reduce its average motion and flexibility, which is often an essential part in the mechanism of an enzyme-substrate reaction. From the data shown above, free Ile 3→Trp has about 78% of WT's activity, and it lost more activity than that of WT after adsorption onto nanoparticles. This is consistent with previous findings by Billsten et al. (1995) and Tian (1996, thesis), that correlated the protein stability with conformational changes upon adsorption. In their studies, Ile 3→ Trp was found to lose about 29% of the α-helix, while WT lost only about 12% of the α-helix. These results indicate that solid surfaces induced conformational changes and activity reduction of T4 lysozymes. The molecular stability of T4 lysozyme can apparently affect both conformational changes and activity reduction.

Table 4.4 The activity of free and adsorbed T4 lysozymes using *E.coli* cell walls as

substrate.

[*]T4-lysozymes	without NP unit/mg	Standard error	with [**]NP unit/mg	Standard error	% [***]0f inactivation
WT	45384	±1593	38926	±1967	14.2%
Trp	35366	±1368	28016	±1600	20.8%

* T4 lysozyme concentration: ranged from 4.8 µg/ml to 33 µg/ml.

**NP - nanoparticle.

*** % of inactivation: (free enzyme activity - adsorbed enzyme activity)/free enzyme

activity.

4.5 Evaluation of T4 lysozyme activity with and without nanoparticles using *M. lysodeikticus.* cell walls

The T4 lysozyme activity was also tested using *M. lysodeikticus* cell walls as a substrate. Representative reaction kinetics graphs of WT, Ile 3→Trp and Ile 3→Cys are shown in figure 4.5a. The standard curves of T4 lysozyme activity before and after adsorption onto nanoparticles are plotted in figure 4.5b and numerically summarized in Table 4.5. The results showed that WT lost part of its activity after adsorption onto nanoparticles, while the activities of Trp and Cys were not measurable after adsorption onto nanoparticles. This is consistent with the results from the *E. coli* cell wall assay, suggesting that the nanoparticle surface induces some degree of conformational change which results in a reduction of activity. Some extent of desorption might occur during adsorption to nanoparticles, but for the adsorption based on 1 : 1 molecular ratio, What we observed is the activities of T4 lysozymes after adsorption were always lower than before adsorption in either *E. coli* cell walls substrate and *M. lysodeikticus* cell walls substrate. Furthermore, previous studies by Billston et al. (1996) and McGuire et al. (1995) indicated that the adsorption of T4 lysozyme to silica particles is irreversible to dilution. So we assumed that only very little desorption occured, and the desorbed molecules might not completely regain their activity because some comformational changes happed after adsorption. From Figure 4.5a and 4.5b, WT again displayed higher activity than its mutants. This may be due to Ile 3→Trp's lower stability and Ile 3→Cys's single disulfide bond which may reduce its motion and limit its productive contact with substrate. Table 4.4 and Table 4.5 showed that the activities of T4 lysozyme using *M.*
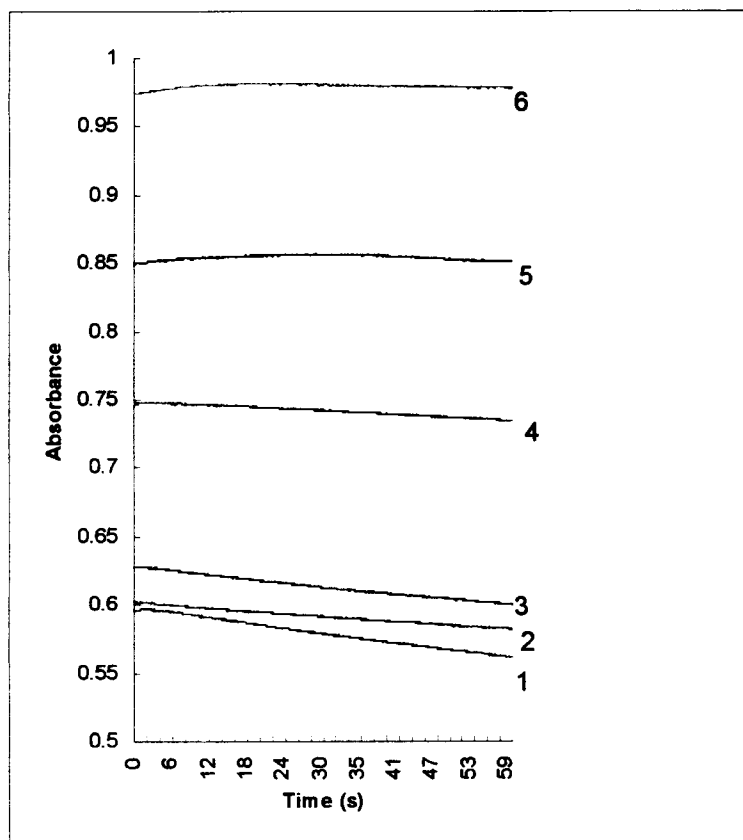
Figure 4.5a Reaction kinetics of WT, Ile 3→Trp and Ile 3→Cys with and without nanoparticles using *M. lysodeikticus* cell walls. Line 1: WT (42μg/ml), line 2: Ile 3→Trp (38μg/ml), line 3: Ile 3→Cys (47μg/ml), line 4: WT (42μg/ml) with NP (nanoparticles), line 5: Ile 3→Trp (38μg/ml) with NP, line 6: Ile 3→Cys (47μg/ml) with NP.
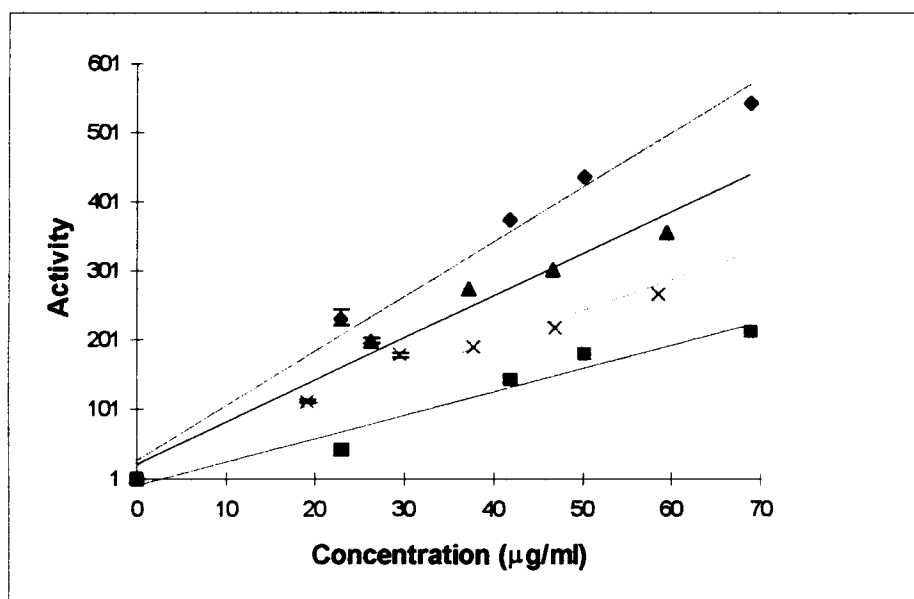
Figure 4.5b The standard curves of WT, WT-nanoparticle complexes, Ile 3→Trp and Ile 3→Cys using *M. lysodeikticus* cell walls as substrate. ◆,WT; ▲, Ile 3→Cys; ×, Ile 3→Trp; ■, WT- nanoparticle complexes. Activity = $mA_{450} \times 10^4/min$.

Table 4.5 The activity of free and adsorbed T4 lysozymes using *M. lysodeikticus* cell walls as substrate.

*T4 lysozymes	without NP** unit/mg	with NP unit/mg	% of inactivation
WT	4260	1427	66.5%
Cys	3290	~	~
Trp	2593	~	~

*T4 lysozyme concentration range from 15 μg/ml to 70 μg/ml.

**NP - Nanoparticle.

lysodeikticus cell walls were about 10 fold lower than the activities of T4 lysozyme using *E. coli* cell walls. This may be due to the substrate specificity, and it is consistent with Tsugita's (1968) study in which found that *E. coli* is the better substrate for T4 phage lysozyme. From our work, the sensitivity of *E. coli* cell wall assay is apparently higher than that of *M. lysodeikticus* cell wall assay (see figure 4.5c).

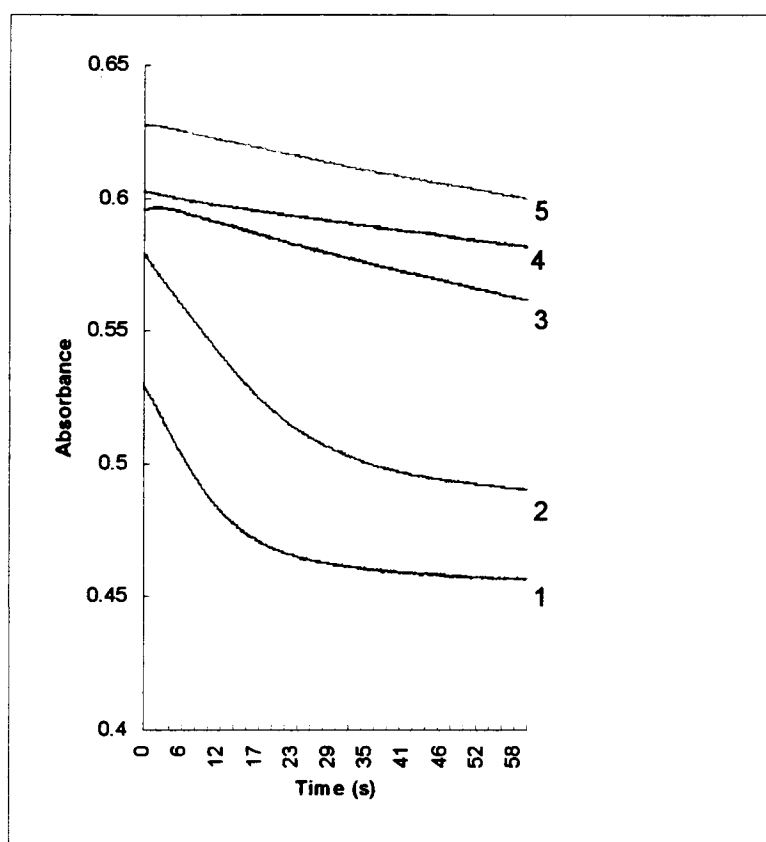Figure 4.5c The reaction kinetics of WT, Ile 3→Trp and their nanoparticle complexes in different substrates. Line 1: WT (24μg/ml)-*E. coli*, line 2: Ile 3→Trp (24μg/ml)-*E. coli*, line 3: WT (42μg/ml)-*M. lysodeikticus*, line 4: Ile 3→Trp (38μg/ml)-*M. lysodeikticus*, line 5: Ile 3→Cys (42μg/ml)-*M. lysodeikticus*.

4.6 Evaluation of T4 lysozyme activity with and without nanoparticles using agar plate assays

The activities of WT, Ile 3→Trp, Ile 3→Cys and their nanoparticle complexes were compared using an agar plate seeded with *M. lysodeikticus* cells. The corresponding diameters of the zone width are shown in Table 4.6. The standard curves of WT, Ile 3→Trp, Ile 3→Cys and their nanoparticle complexes are shown in figure 4.6.

Table 4.6 The diameters of the zone width of T4 lysozyme variants and their nanoparticle complexes after 24 hours incubation.

T4 lysozyme variants (0.5mg/ml)	Diameter without NP (cm)	Diameter with NP (cm)
Cys	1.5	1.4
WT	1.3	1.2
Trp	1.2	1.1

Both Table 4.6 and figure 4.6 showed that the activity of T4 lysozyme was affected by the adsorption onto nanoparticles. The zone diameters of T4 lysozyme variants measured after adsorption onto nanoparticles were smaller than that free T4 lysozyme variants, which means part of the activity was lost after adsorption. These results again confirm that nanoparticle surfaces induce T4 lysozyme activity reduction. The results also showed that the Ile 3→Cys variant has a bigger zone width than those of WT regardless of whether the nanoparticles were present. This might be due to its higher
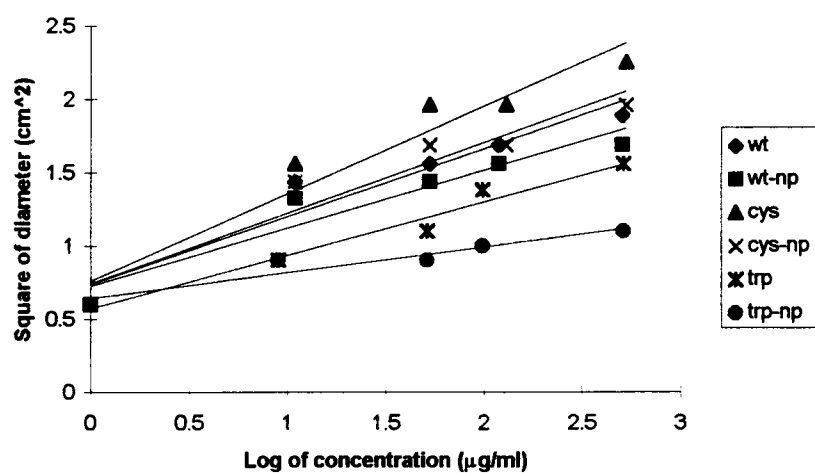
Figure 4.6 The standard curves of WT, Ile 3→Trp, Ile 3→Cys and their nanoparticle complexes measured by agar plate bioassays. Ile 3→Cys (▲), Ile 3→Cys with NP (×), WT (◆), WT-NP (■), Ile 3→Trp (✳),Ile 3→Trp with NP (●).

stability and less conformational changes during adsorption (Billston et al., 1996). A previous study revealed that Ile 3→Cys doesn't bind tightly to silica particles (McGuire et al., 1995), this might be another factor that Ile 3→Cys has higher activity retention. Also, it is possible that Ile 3→Cys desorbed from the silica surfaces after the long time incubation (24hr) and regained more enzymatic activity than those WT and Ile 3→Trp. From the results, it is apparent that the sensitivity of agar plate bioassays is lower than that of the spectrophotometric techniques, but its replication was very good.

In this study, we have presented quantitative data on the structural effects of enzymatic activity of T4 lysozyme upon adsorption onto colloidal silica surfaces. The results enable us to compare the enzymatic activities of different T4 lysozyme stability mutants before and after adsorption. The data suggests that the adsorbed protein is partially functional. The extent of activity reduction was different from the stability of each protein. Proteins with lower stabilities have a greater tendency to lose their activity. It should be noted that the temperature and the pH as well as the OD (optical density) of the substrates are very critical in each assay. For a good comparison, different stability mutants have to be produce in the same period to prevent protein denaturation. The substrates need to be freshly prepared and the OD of the substrates should be as close as possible. Assays should be performed at the same time to minimize the error. Because of the limitations of past experiments, further study of enzymatic activity upon adsorption using sugar reducing assay with *E. coli* cell walls as substrate is recommended.

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