

Bi-functional Activities of Chimeric Lysozymes Constructed by Domain Swapping between Bacteriophage T7 and K11 Lysozymes

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The lysozymes encoded by bacteriophage T7 and K11 are both bifunctional enzymes sharing an extensive sequence homology (75%). The constructions of chimeric lysozymes were carried out by swapping the N-terminal and C-terminal domains between phage T7 and K11 lysozymes. This technique generated two chimeras, T7K11-lysozyme (N-terminal T7 domain and C-terminal K11 domain) and K11T7-lysozyme (N-terminal K11 domain and C-terminal T7 domain), which are both enzymatically active. The amidase activity of T7K11-lysozyme is comparable with the parental enzymes while K11T7-lysozyme exhibits an activity that is approximately 45% greater than the wild-type lysozymes. Moreover, these chimeric constructs have optimum pH of 7.2-7.4 similar to the parental lysozymes but exhibit greater thermal stabilities. On the other hand, the chimeras inhibit transcription comparable with the parental lysozymes depending on the source of their N-terminals. Taken together, our results indicated that domain swapping technique localizes the N-terminal region as the domain responsible for the transcription inhibition specificity of the wild type T7 and K11 lysozymes. Furthermore, we were able to develop a simple and rapid purification scheme in purifying both the wild-type and chimeric lysozymes.

Keywords: Amidase, His-tagging, K11 RNA polymerase, Phage K11 lysozyme, Transcription inhibition

In this paper N-terminal histidine tagged K11, T7 and chimeric lysozymes by swapping the N-terminal and C-terminal domains between T7 and K11 lysozymes were constructed. These lysozymes were purified and characterized.

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Introduction

The bacteriophage group that includes T7 and K11 produces two important proteins that have been widely used as models for the investigation of the physicochemical aspect of transcription. These proteins are the DNA-dependent RNAP (RNA polymerase) (Chamberlin *et al.*, 1970; Dietz *et al.* 1990) and lysozyme (Inouye *et al.*, 1973; Junn *et al.*, 2005). The bacteriophage RNAPs are single-subunit proteins that are able to perform the complete transcriptional cycle in the absence of additional protein factors and show stringent specificity toward their promoters (Rosa and Andrews, 1981; Dunn and Studier, 1983; Brown *et al.*, 1986; Han *et al.*, 2002). On the other hand, the lysozymes encoded by bacteriophage T7 and K11 are both bifunctional enzymes. They possess amidase activity and bind specifically to their respective RNAPs and inhibit transcription (Ikeda and Bailey, 1992; Cheng *et al.*, 1993; Huang *et al.*, 1999; Junn *et al.*, 2005) and T7 lysozyme stimulates replication and packaging of T7 DNA (Zhang and Studier, 2004).

The bacteriophage T7 lysozyme has already been extensively studied. Its x-ray crystallographic data revealed that T7 lysozyme contains a zinc atom necessary for amidase activity but not for inhibition of T7 RNAP (Junn *et al.*, 2005). The crystal structure of T7 lysozyme-T7 RNAP complex has also been elucidated revealing that lysozyme binds at a site remote from the polymerase active site suggesting an indirect mechanism of inhibition (Jeruzalmi and Steitz, 1998; Junn *et al.*, 2005). Also, mutational analysis identified that the N terminus of T7 lysozyme gene appeared to be important in interacting with T7 RNAP (Cheng *et al.*, 1994). T7 RNAP mutants that have an increased or decreased sensitivity to lysozyme inhibition have been isolated during T7 phage infection (Moffatt and Studier, 1987; Zhang and Studier, 1995; Zhang and Studier, 2004). The complex between T7 lysozyme and T7 RNAP is essential and tightly regulated in

phage growth (Moffatt and Studier, 1987). Thus, mutant T7 RNAP or lysozyme unable to form the complex were isolated either by direct engineering or as suppressors (increased phage production) of hypersensitive polymerase mutants or over production of lysozyme (Zhang and Studier, 1995).

However, structural and functional studies on K11 lysozyme are relatively limited. The lysozyme gene of bacteriophage K11 was recently sequenced in our lab and the deduced amino acid sequence of its polypeptide shows 75% homology with the T7 lysozyme (Junn *et al.*, 2005). The N-terminus of T7 lysozyme and the amino acid residues 30 to 42 which are found to be the site of interaction with T7 RNAP are conserved in K11 lysozyme. Moreover, Lys¹²⁸ and Tyr⁴⁶, which are important for amidase activity, are also conserved in K11 lysozyme. The highly conserved amino acid residues in the N-terminal and essential sites of T7 and K11 lysozyme genes suggest that both lysozymes have the same functional role in the bacteriophages (Junn *et al.*, 2005).

In this study, we constructed a phage K11 lysozyme fused to a hexahistidine tag to facilitate single-step purification directly from the crude cell lysates. This method aimed to provide sufficient amount of protein for routine biochemical analysis. Although this technique is extremely useful for the rapid purification of lysozyme, the purification tag inevitably alters the primary sequence of the protein since two amino acid residues remain even after the tag is removed. We have therefore examined the effect of this tag on the amidase and RNAP inhibition activity of the enzyme. Moreover, since the two enzymes share a relatively high homology (75%) and contain conserved essential sites (Junn *et al.*, 2005), we also constructed chimeric enzymes by swapping the N-terminal and C-terminal domains between T7 and K11 lysozymes. The characterization of such chimeras would provide further insight into the effect of swapping and the role of each domain on amidase activity and RNAP inhibition activity of the lysozymes.

Materials and Methods

Construction of hexahistidine-tagged wild-type K11 and T7 lysozymes. Using pET-tacKLys (Junn *et al.*, 2005) and pLyse (purchased from Novagen) plasmids as templates, 450 bp fragments containing the K11 and T7 lysozyme genes were amplified by polymerase chain reaction using Taq-EF DNA polymerase (purchased from Takara) respectively. The sequences of the forward and reverse primers for K11 lysozyme gene are 5'-GGAATTCCATATGGCCAAGGTTCAATTCA-3' and 5'-GCGGATCCCTAATGTTTACCCACGGT-3' and those for T7 lysozyme gene are 5'-GGCGCATATGGCTCGTGTACAGTTT-3' and 5'-GCGGATCCTTATCCACGGTCAGAAGT-3', respectively. They were designed to anneal to upstream and downstream flanking sequences just outside the lysozyme gene and to contain *NdeI* and *BamHI* recognition sequences (underlined). The resulting PCR fragments were digested with *NdeI* and *BamHI* restriction enzymes and were inserted into the *NdeI/BamHI* sites of cleaved expression vector

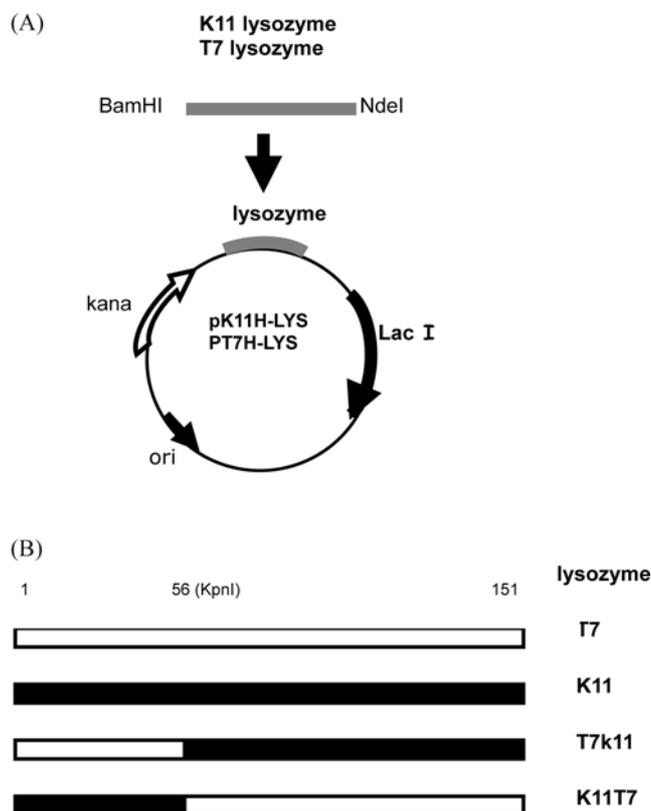


Fig. 1. (A) Structure of expression vectors for the N-terminal histidine tagged K11 and T7 lysozyme genes. The pK11H-LYS and pT7H-LYS plasmids contain the hexahistidine tagged K11 and T7 lysozyme gene, respectively, under the control of *lac* promoter. They also carry the *bla* gene for ampicillin resistance and the *lacI* gene for the *lac* repressor. (B) Construction of chimeric lysozymes of swapping the N-terminal and C-terminal domains between T7 and K11 lysozymes.

pET28a (purchased from Novagen) and the resulting recombinant plasmids were named pK11H-LYS for K11 lysozyme and pT7H-LYS for T7 lysozyme (Fig. 1A).

Construction of domain swapping mutants. To swap the N-terminal and C-terminal domains between T7 and K11 lysozymes, a *KpnI* restriction site was introduced in T7 lysozyme by site-directed mutagenesis using overlap extension PCR method. Two outside primers are same primers used for the construction of pT7H-LYS and each contains *NdeI* and *BamHI* restriction site respectively. Two internal primers with mismatched base pair sequence (in bold), forward primer 5'-CAAGCGAGACGGTACC GTGGAGGCAG-3' and reverse primer 5'-CCTCCACGGTACCG TCTCGCTTGATG-3', were designed to introduce a single base substitution thus generating a *KpnI* restriction site (GGTACC) at the position where the same restriction site is present in the nucleotide sequence of K11 lysozyme. DNA sequencing confirmed that the necessary substitution was incorporated in the T7 lysozyme gene.

To construct the chimeric lysozymes, the pK11H-LYS vector containing the wild type K11 lysozyme and the PCR-generated mutant T7 lysozyme were both digested with *KpnI* and *NdeI*

restriction enzymes. The T7 lysozyme fragment (168 bp) containing the N-terminal domain of the enzyme was then inserted and cloned into the cleaved pK11H-LYS generating the T7K11-lysozyme mutant. This chimeric enzyme contains 56 amino acids of T7 lysozyme N-terminal and 95 amino acids of K11 lysozyme C-terminal (Fig. 1B). Accordingly, to generate the other domain swapping mutant, the pK11H-LYS vector and the mutant T7 lysozyme was cleaved with *KpnI* and *BamHI* restriction enzymes. The T7 lysozyme fragment (285 bp) generated in this restriction digestion was cloned into the previously digested pK11H-LYS generating the K11T7-lysozyme mutant. This domain swapped enzyme contained 56 amino acids of K11 lysozyme N-terminal and 95 amino acids of K11 lysozyme C-terminal (Fig. 1B).

Expression and purification of hexahistidine-tagged K11 and chimeric lysozymes. *E. coli* BL21 (DE3) cells were transformed respectively with pK11H-LYS, pT7K11H-LYS, and pK11T7H-LYS and grown on LB plates containing kanamycin (50 µg/ml). Overnight cultures in 5 ml LB broth were started with single colonies and used to inoculate expression cultures (200 ml). When the culture reached $A_{600}=0.5$, IPTG was added to a final concentration of 0.1 mM and cultivation was allowed to proceed for 4 h at 37°C.

After induction, cells were harvested by centrifugation and resuspended in buffer A containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 5% glycerol. The cells were then sonicated on ice using a microtip with 50% power for five 30-s interval and soluble and insoluble fractions were separated by centrifugation. The pellet fraction was stored at -80°C until use. Whole cell lysates, induced and uninduced, and soluble and insoluble fractions were analyzed by 15% SDS-PAGE and bands were visualized by Coomassie blue staining.

The hexahistidine-tagged K11 and chimeric lysozyme proteins were purified under denaturing conditions using Ni-NTA affinity chromatography. All steps were carried out at 0-4°C. Frozen pellet cells were thawed on ice and resuspended in 50 ml of buffer containing 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, and 7 mM β-mercaptoethanol, and repelleted by centrifugation (15,000 rpm for 30 min). After washing twice using this procedure, the pellet was solubilized with 20 ml of Solution A containing 8 M urea, 10 mM Tris-Cl, pH 8.0, and 100 mM Na-phosphate and was incubated on ice with gentle shaking for 1 h. Insoluble materials were removed by centrifugation (15,000 rpm for 30 min).

Three milliliters of Ni-NTA agarose (purchased from Qiagen) was added to the clarified supernatant and the mixture was gently shaken on ice for 1 h to facilitate binding. The mixture was then loaded on an empty column and the unbound protein was eluted. Nonspecific proteins that bound with the Ni-NTA agarose were eluted by washing the column with 100 ml of Solution A at pH 6.3. The hexahistidine-tagged K11 and chimeric lysozymes were finally eluted with 10 ml of Solution A at pH 4.5. The fractions were examined by 15% SDS-PAGE and the appropriate fractions were pooled and refolded by dialyzing against a buffer containing 25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 M urea, 7 mM β-mercaptoethanol, and 20% glycerol. After a 3-h dialysis using this solution, the proteins were transferred and dialyzed overnight against a buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 7 mM β-mercaptoethanol, and 20% glycerol. The N-terminal tag of

the lysozymes was cleaved by adding 20 µl of thrombin (purchased from Sigma) and incubating at 4°C for 22 h. The cleaved tag and thrombin were removed by passing the reaction mixture in 200 µl of DEAE resin. The purified thrombin cleaved protein was stored in aliquots at -20°C until use for amidase and *in vitro* transcription assays.

Expression and purification of hexahistidine-tagged wild-type T7 lysozyme. *E. coli* BL21 (DE3) cells were transformed with pT7H-LYS containing HIS-tagged T7 lysozyme gene and grown on LB plates containing kanamycin (50 µg/ml). The cultivation and induction procedures of K11 lysozyme gene described above were also used for the cultivation and expression of T7 lysozyme gene.

The histidine tagged T7 lysozyme was mostly found in the soluble supernatant fraction thus it was purified under non-denaturing conditions using Ni-NTA affinity chromatography. The soluble fraction was mixed with 3 ml of Ni-NTA agarose that had been previously washed and equilibrated with buffer containing 50 mM Tris-Cl (pH 8), 300 mM NaCl, and 20 mM imidazole. The reaction mixture was gently shaken on ice for 1 h to facilitate binding. The mixture was then poured into an empty column and the unbound fraction was eluted. The resin was then washed with 100 ml of the previous buffer to remove nonspecific binding. The T7 lysozyme was finally eluted with elution buffer containing 50 mM Tris-Cl (pH 8), 300 mM NaCl and 250 mM imidazole. The appropriate protein fractions were then pooled and dialyzed against a buffer containing 50 mM Tris-Cl, 100 mM NaCl, 0.1% Triton-X, 7 mM β-mercaptoethanol, and 50% glycerol. The purified T7 lysozyme was cleaved with thrombin and was passed through a DEAE resin. The purified thrombin cleaved protein was stored in aliquots at -20°C until use for amidase and *in vitro* transcription assays.

Amidase assay. The amidase activity was assayed by measuring the decrease in turbidity of *E. coli* XL-1 blue cells that were incubated in 0.1 M EDTA, pH 8.0 for 5 min at room temperature, pelleted, and resuspended in PBS buffer and kept in ice until use (Cheng *et al.*, 1993; Junn *et al.*, 2005). To measure cell wall lysis activity, various concentrations of phage lysozymes, were added to 1 ml of sensitized cells that had been brought to room temperature and the decrease in OD₆₀₀ was monitored. For amidase assay at elevated temperatures, the enzyme was incubated for 10 min at various temperatures. One unit of activity was defined as the amount of enzyme that reduced OD₆₀₀ at the rate of 0.001/min.

***In vitro* transcription assay.** A 20 µl standard transcription reaction containing 40 mM Tris-Cl pH 8, 8 mM MgCl₂, 20 mM spermidine, 10 mM DTT, 0.5 mM NTP, 2 µCi of [α -³²P]CTP (30 µCi/µM), 0.1 µg of template DNA, 10 U of T7 or K11 RNAP purified using the method previously described (Han *et al.*, 1999), and variable amounts of lysozyme was used. The reactions were initiated by adding the RNAP and the reaction mixtures were incubated at 37°C for 30 min. The reaction were terminated by adding 10% TCA solution and the mixtures were incubated on ice for 10 min. The solution was then filtered and the amount of RNA synthesized was determined by measuring the amount of radioactivity retained on the GF/B filter (purchased from Whatman) by a Beckman multi-purpose Scintillation counter LS 6500.

Results

Cloning and expression of hexahistidine-tagged K11, T7 and chimeric lysozymes. The bacteriophage K11 lysozyme gene was amplified by PCR using pK11H-LYS as template. The amplified lysozyme gene, which was engineered to contain *Nde*I and *Bam*HI recognition sequences, was cloned into a pET-28a vector under the control of T7 promoter.

The wild type K11 lysozyme has a *Kpn*I (GGTACC) recognition sequence at bp 164-169 which encodes the amino acids Gly⁵⁵ and Thr⁵⁶. These amino acid residues are conserved in T7 lysozyme but Thr⁵⁶ is encoded by a different codon thus the corresponding nucleotide sequence for T7 lysozyme is GGTACT. To introduce a *Kpn*I site in T7 lysozyme, we employed site-directed mutagenesis by overlap PCR to generate a single base substitution (GGTACT to GGTACC). This procedure introduced one silent point mutation in the coding region of T7 lysozyme changing ACT triplets encoding Thr⁵⁶ to the synonymous codon ACC. The lysozyme genes were then cut in this region by *Kpn*I restriction enzyme digestion and their respective domains were swapped (Fig. 1B). Two chimeric enzymes were generated, one which contains a T7 N-terminal and a K11 C-terminal and another which contains a K11 N-terminal and T7 C-terminal and were named T7K11-lysozyme and K11T7-lysozyme, respectively. Cloning of each chimera was confirmed by cutting with restriction enzyme unique to each swapped domain; *Afl*III for K11T7-lysozyme and *Dra*I for T7K11-lysozyme.

E. coli BL21 (DE3) cells harboring pK11H-LYS were cultured at 37°C in the presence and absence of IPTG and the whole cell lysates were analyzed by 15% SDS-PAGE as shown in Fig. 2A. One major band appeared at approximately 21 kDa, a size slightly higher than the actual protein size since the lysozyme is tagged and carries 15 additional amino acid residues on its N-terminal. Although the lysozyme was efficiently expressed at 37°C, almost all of the lysozyme fractions aggregated as inclusion bodies showing the same results of previously reported in our lab (Junn *et al.*, 2005). On the other hands, T7 lysozyme is induced at 37°C and mostly found in the soluble supernatant fractions (Fig. 2A). Also, both of the chimeric lysozymes are induced at 37°C and most lysozyme fractions are aggregated as inclusion bodies (Fig 2A).

Inclusion body formation is a common problem encountered in expressing recombinant proteins in *E. coli*. Several alterations of the expression protocol were tried in increasing the amount of soluble protein. Decreasing the induction temperature did not produce a substantial amount of soluble lysozyme (data not shown).

A possible strategy for the prevention of protein aggregation is the co-expression of molecular chaperones. We co-transformed the plasmid containing the lysozyme gene with pGroESL, a plasmid containing the GroES and GroEL genes which are known to enhance protein solubility. However, this technique

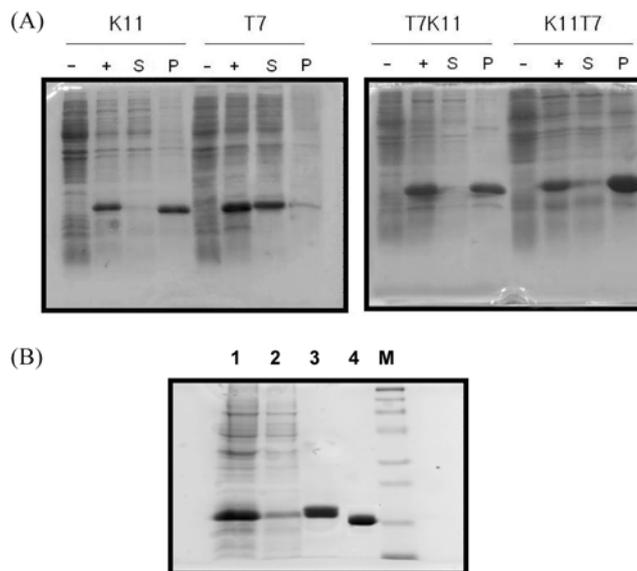


Fig. 2. (A) SDS-PAGE analysis of K11, T7 and chimeric lysozymes. K11, T7, T7K11, K11T7 lysozymes are induced at 37°C. Their genes were either induced (+) or uninduced (-) with IPTG; soluble (S) and insoluble (P) fractions were separated by centrifugation. Equal amounts of protein were loaded on each lane. (B) Purification of hexahistidine-tagged K11 lysozyme. The inclusion bodies were solubilized in 8 M urea and purified in Ni-NTA column under denaturing condition. Lane 1, inclusion bodies solubilized with 8 M urea; lane 2, Ni-NTA unbound fraction; lane 3, Ni-NTA elution fraction; lane 4, cleaved lysozyme; lane M, protein size marker

again failed to prevent aggregation and to promote proper folding since most of the lysozyme remained in the pellet fraction (data not shown). The insolubility of K11 lysozyme in *E. coli* might have been caused by its difference in amino acid sequence and in its natural host (*Klebsiella* for phage K11) (Junn *et al.*, 2005). Moreover, it seemed to be likely that K11 lysozyme belongs to a group of proteins that fold improperly upon intracellular expression in *E. coli*.

Purification of hexahistidine-tagged K11, T7 and chimeric lysozymes. Since various techniques in producing soluble lysozyme did not give favorable results, the protein was purified from inclusion bodies. The pellet was solubilized in 8 M urea and was fractionated by Ni-affinity chromatography under denaturing condition. The purification of K11 lysozyme is summarized in Table 1. Approximately 13.5 mg of homogenous K11 lysozyme was obtained from a 200-ml cell culture. Fractions of eluted protein were analyzed by 15% SDS-PAGE and stained with Coomassie blue. As shown in Fig. 2B, approximately 97% homogenous K11 lysozyme was recovered in a single-step elution. The elution fractions resulted in a single protein band. Thus, with the use of hexahistidine tag, we were able to develop a simple and rapid purification for K11 lysozyme to be used for further

Table 1. Purification of tagged bacteriophage K11 lysozyme

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Protein yield (%)
Crude extract	620	-	-	100
Inclusion body	152	-	-	24.5
Ni-NTA elute	13.5	19,400	1,437	2.1

biochemical characterization of the enzyme.

Refolding of the denatured lysozyme was carried out by dialysis with a buffer containing an intermediate denaturant concentration (Clark, 1998). Previous refolding of K11 lysozyme resulted in aggregation (Junn *et al.*, 2005); however, with the presence of 2 M urea in the dialysis buffer, we were able to refold the lysozyme without any observed aggregation. This implied that the denaturant concentration was high enough to solubilize aggregates but low enough to promote proper folding. The protein solution was then transferred to the same buffer without the presence of the denaturant and dialysis was continued until the protein refolded completely.

The relative specific activity of the purified lysozyme was estimated to be 1437 U/mg (Table 1). Compared to the previously purified wild-type lysozyme in our laboratory; this activity is about 60% higher. Previously purified K11 lysozyme without hexahistidine was reported to have a specific activity of 900 U/mg (Junn *et al.*, 2005). The observed increase in enzyme activity might be attributed in the presence of the tag. Affinity tags can be helpful for stabilizing proteins or enhancing their solubility (Terpe, 2003). Even upon cleaving the hexahistidine tag, the N-terminal of the lysozyme still has two extra amino acid residues (Gly-Ser) which, based on our results, can still exert the same effect.

Both chimeras were efficiently purified by using the same purification method of K11 lysozyme but the protein yield of K11T7-lysozyme was lower compared with T7K11-lysozyme and the parental lysozymes. A 200-ml cell culture yielded 13.1 mg T7K11-lysozyme while similar amount of culture yielded 9.3 mg K11T7-lysozyme. The protein yield of wild type T7 and K11 lysozymes is 16.5 mg and 13.5 mg, respectively (data not shown). Differences in purification profiles may be due to modifications of the enzyme conformations caused by the interaction of domains derived from the different wild type proteins (Berger *et al.*, 2001). The homology between T7 and K11 lysozymes corresponding with the swapped domain in N-terminal part (1-56 amino acids) is 78% while in C-terminal part (57-151 amino acids) is only 66%.

Amidase activities of wild type and chimeric lysozymes. To determine whether the purification tag affects the enzymatic function of the protein, purified lysozyme fractions with tag and with cleaved tag were assayed as described in Materials and Methods. The amidase activity of tagged lysozyme and cleaved lysozyme were almost identical implying that the

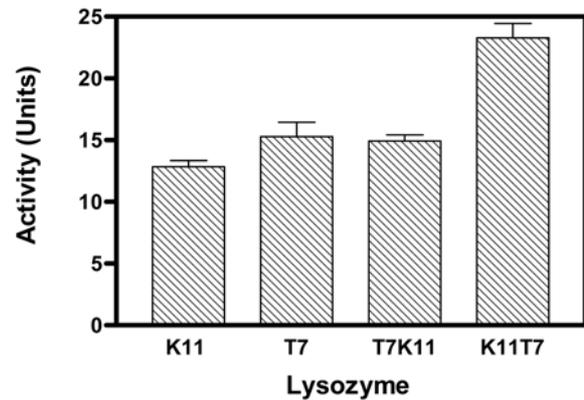


Fig. 3. Amidase activities of K11, T7 and chimeric lysozymes. The amidase activity was assayed by measuring the decrease in turbidity of *E. coli* XL-1 cell.

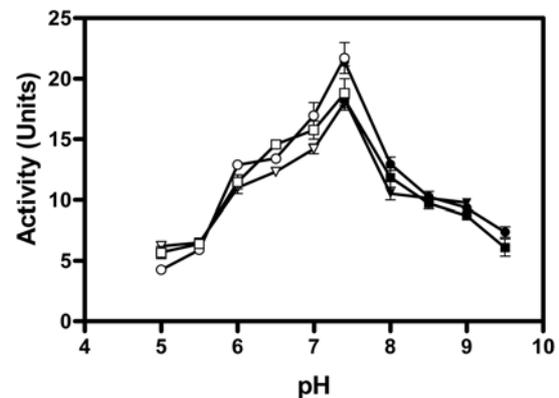


Fig. 4. pH profile of cell wall lysis activity of K11 and chimeric lysozymes. Closed symbol shows the activities measured in Tris-Cl buffer (pH 7.2-9.0) and open symbol shows the activities measured in PBS buffer (5.0-7.4). The amidase activities of K11 (∇ and \blacktriangledown), T7K11 hybrid (\square and \blacksquare) and K11T7 (\circ and \bullet) hybrid were shown.

purification tag did not interfere with the enzyme function (data not shown). This result also suggests that the refolding process restored the activity of the protein.

The amidase activity of the chimeric lysozymes was assayed as previously described for wild type lysozymes (Cheng *et al.*, 1993). As shown in Fig. 3, both chimeras possess amidase activities. The activity of T7K11-lysozyme was comparable with the activities of wild type T7 and K11 lysozymes. On the other hand, the K11T7-lysozyme chimera exhibited a much higher activity that is approximately 45% greater than the parental lysozymes.

We also examined the effect of pH on the enzymatic activity of the protein. Sensitized *E. coli* XL-1 blue cells were resuspended in different buffers at various pH; PBS buffer at pH 5.0-7.4; and Tris-Cl buffer at pH 7.2-9.0. The optimal pH for the enzyme activity of the purified wild type lysozyme was observed at pH 7.2 to 7.4 (Fig. 4). The optimum pH

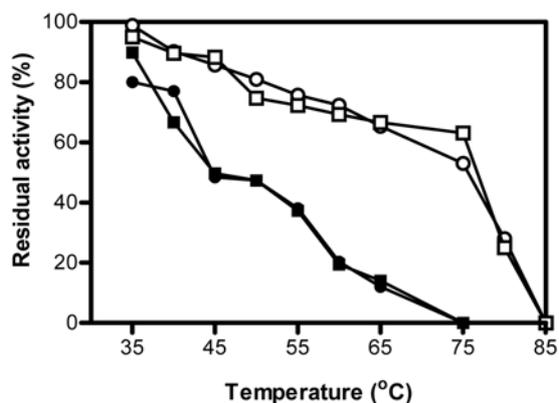


Fig. 5. Thermal stability of wild and chimeric lysozymes at various temperature. After incubation for 10 min at each temperature, cell wall lysis activities of K11 (●), T7 (■), T7K11 (□) and K11T7 (○) lysozymes were measured.

observed for both chimeric lysozymes is similar to that of the parental lysozymes which is 7.2-7.4 (Fig. 4). Bacterial cell wall degradation at acidic or basic pH was also observed but the activity of the enzyme at these conditions was minimal.

The stability of the enzyme at elevated temperatures is shown on Fig. 5. The lysozyme fraction was incubated for 10 min at each indicated temperature and amidase activity was monitored. The amidase activity of the heat-treated lysozyme with hexahistidine tag was found to be more stable than the lysozyme with cleaved tag (data not shown). However, the chimeric lysozymes exhibited thermal stabilities significantly greater than the parental enzymes (Fig. 5). At 50°C, the amidase activities of both wild type K11 and T7 lysozymes are decreased to 47% and 48%, respectively. However, with the same temperature, the T7K11-lysozyme retained 75% activity while the K11T7-lysozyme retained 81% activity. The parental lysozymes are both completely inactivated at 75°C while the chimeric lysozymes lost their amidase activities at 85°C.

Transcription inhibition activities of wild type and chimeric lysozymes. The activities of T7 and K11 RNAPs were determined by measuring the conversion of radiolabeled NTPs into high molecular weight RNA that can be retained on GF/B Whatman filters (Ikeda and Bailey, 1992). Using this filter binding assay, the transcription of T7 and K11 DNA by their respective polymerase was monitored in the presence of increasing amounts of wild type and chimeric lysozymes.

Results from previous experiments revealed that phage T7 and K11 transcription systems are crossly regulated by their own lysozymes (Junn *et al.*, 2005). That is, a certain extent, transcription by T7 RNAP could be regulated by K11 lysozyme and transcription by K11 RNAP could be regulated by T7 lysozyme. However, results also showed that these inhibitions are less compared with their respective lysozymes implying that inhibition is specific (Ikeda and Bailey, 1992; Junn *et al.*, 2005).

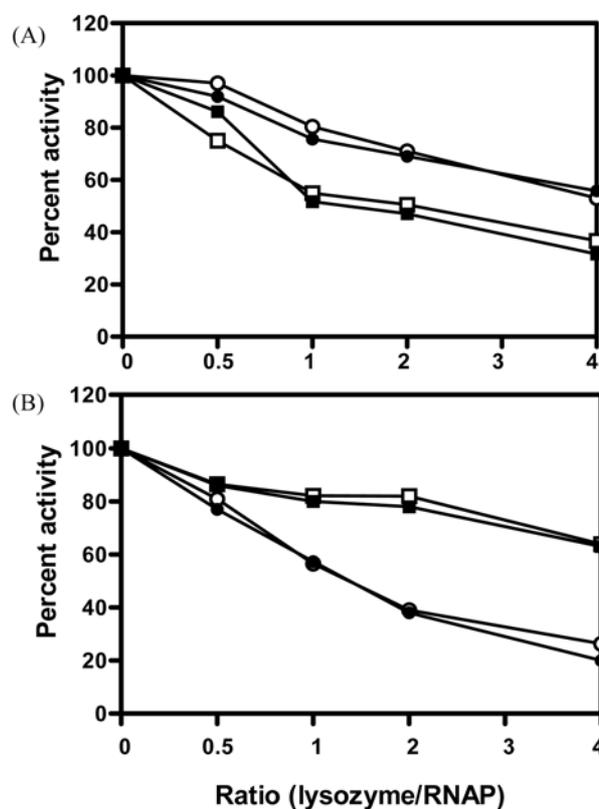


Fig. 6. (A) Inhibition of T7 RNA polymerase transcription by wild-type and chimeric lysozymes. One hundred percent activity for the T7 RNA polymerase is defined as the transcriptional activity measured in the absence of lysozyme. In the increasing ratio of K11 (●), T7 (■), T7K11 (□) and K11T7 (○) lysozymes to T7 RNAP, the relative transcription activities were measured respectively. (B) Inhibition of K11 RNAP transcription by wild-type and chimeric lysozymes. One hundred percent activity for the K11 RNAP is defined as the transcriptional activity measured in the absence of lysozyme. In the increasing ratio of K11 (●), T7 (■), T7K11 (□) and K11T7 (○) lysozymes to K11 RNAP, the relative transcription activities were measured respectively.

The transcription inhibition activities of the wild type and chimeric lysozymes are shown on Fig. 6. With transcription by T7 RNAP, the T7K11-lysozyme exhibited inhibitory effect that is almost identical with the wild-type T7 lysozyme. At 1-fold excess of T7 lysozyme and T7K11 lysozyme, transcription with T7 RNAP is decreased to 52% and 55%, respectively. Similarly, with transcription by K11 RNAP, the K11T7-lysozyme showed transcription inhibition that is comparable with wild type K11 lysozyme. At equimolar concentrations of K11 RNAP and K11 lysozyme, transcription activity is decreased to 57% while a 1-fold excess of K11T7-lysozyme decreased transcription to 56%.

Studier *et al.* reported earlier that the N-terminus of T7 lysozyme seems to be likely the site of interaction with T7 RNAP (Cheng *et al.*, 1993). Since the T7K11-lysozyme

contained the N-terminus of T7 lysozyme, it could be expected that it would exhibit inhibitory effect similar to the wild-type T7 lysozyme. The comparable inhibitory activities of K11 lysozyme and K11T7-lysozyme suggest that like T7 lysozyme, the N-terminus of K11 lysozyme could also be the site of interaction with K11 RNAP. However, it would be very interesting to note that the N-terminal of both wild type lysozymes share a high degree of homology (80%) but are nonetheless specific in inhibiting transcription by their respective RNAPs.

Discussion

Routine biochemical characterization of enzymes often requires rapid and simple purification scheme. Isolation of proteins with histidine tag is the most commonly used method because of its relatively small size and charge of the polyhistidine affinity tag ensure that protein activity is rarely affected (Terpe, 2003). We are interested in the characterization of phage K11 lysozyme, a bifunctional enzyme homologous with the widely studied T7 lysozyme. In this study, we described the construction, expression and purification of hexahistidine-tagged phage K11 lysozyme. Our results indicated that the purification tag did not affect the enzymatic activities of K11 lysozyme. The amidase activity of the tagged K11 lysozyme is approximately 55% greater than the previously purified non-tagged lysozyme (Junn *et al.*, 2005). This result might be attributed in the presence of the tag that could be helpful in stabilizing the enzyme. Previous purification of K11 lysozyme in our laboratory resulted in extensive aggregation and this might affect amidase activity. On the other hand, its ability to inhibit transcription by RNAP *in vitro* is almost identical with the non-tagged lysozyme. Previously, a hexahistidine-tagged T7 lysozyme was purified and characterized in our laboratory and results also revealed that tagged T7 lysozyme possessed activities that are almost indistinguishable with the non-tagged enzyme. Considering these results, we assumed that utilization of such purification tag would likewise be tolerated in the construction of chimeric enzymes between the two phage lysozymes. Thus, we exploited this affinity in expressing and purifying the chimeric constructs.

The lysozymes encoded by phage T7 and K11 show a high degree of homology (75%). Both are bifunctional enzymes that exhibit amidase activity and transcriptional inhibitory activity which are not separated into independent domains. The amidase activities of T7 and K11 lysozymes are comparable using *E. coli* as substrate. However, their ability to inhibit transcription *in vitro* is specific with their respective RNAPs. Another difference between T7 lysozyme and K11 lysozyme is their solubility upon expression in *E. coli*. T7 lysozyme is mostly found on soluble fractions but K11 lysozyme aggregates as inclusion bodies. The insolubility of K11 lysozyme in *E. coli* may have been caused by its

difference in amino acid sequence and in natural host (*Klebsiella* for the phage K11) as the case of k11 RNAP (Han *et al.*, 1999)

Domain swapping between T7 and K11 lysozymes generated two chimeras that were both efficiently overexpressed in *E. coli* and enzymatically active. However, both chimeras were mostly expressed as inclusion bodies. The T7K11-lysozyme chimera exhibited amidase activity that is almost identical with the parental enzymes while the K11T7-lysozyme showed an increased activity that is approximately 45% greater than parental enzymes. The increased amidase activity of K11T7-lysozyme could be due to a more favorable structure adapted by the chimera that allows a more efficient binding of the amidase substrate. Furthermore, although both chimeras have the same optimum pH as the wild-type enzymes, they showed a significant variation in thermal stabilities from their parent enzymes. At 75°C, the residual amidase activity of T7K11-lysozyme and K11T7-lysozyme is 63% and 53%, respectively but the parental enzymes are completely inactivated at this temperature. Inactivation of the chimeras was observed at 85°C. Generally, hydrophobic interaction inside a protein molecule is an important factor in stabilizing its structure. Heat stability may be influenced by only a few amino acids substitutions (Singh and Hayashi, 1995). The thermal stability of the chimeras might be possibly due to a significant increase in their hydrophobic properties. Hence, prediction of their structures could provide more insight on their improved properties.

It was previously reported that the N-terminus of T7 lysozyme is the site of the interaction with T7 RNAP and deletion mutant lacking amino acid residues 2-5 was found to be completely deficient in binding and inhibiting transcription while retaining full amidase activity (Cheng *et al.*, 1993). The chimeras showed transcription inhibition activities that are similar to the parent lysozymes depending on the source of their N-terminal. The T7K11-lysozyme containing a T7 N-terminal inhibits transcription that is comparable with T7 lysozyme. On the other hand, K11T7-lysozyme, which contains a K11 N-terminal showed the same magnitude of transcription inhibition as K11 lysozyme. The inhibitory effect exhibited by the chimeras implies that the N-terminal of the parental lysozymes is responsible for transcription inhibition specificity. Since it was already established that the N-terminal of T7 lysozyme is the site of binding with T7 RNAP, these results confirmed our previous assumption that the N-terminal of K11 lysozyme is also the binding site of K11 RNAP.

In summary, we were able to construct vectors that efficiently express hexahistidine tagged phage K11 lysozyme and chimeric lysozymes. We were also able to develop a simple method in purifying these enzymes directly from the crude cell lysates using Ni²⁺ affinity chromatography. Domain swapping technique demonstrated that the N-terminals of T7 and K11 lysozymes determine their specificity with respect to their ability to inhibit transcription by phage RNAP *in vitro*.

On the other hand, their amidase activity is not localized on the swapped region and amidase binding could be somewhat dependent on critical residues that are located randomly within the enzyme. These residues are identified in T7 lysozyme and conserved in K11 lysozyme. Site-directed mutations on K11 lysozyme at these sites, therefore, could provide a better understanding on its structure and amidase activity relationship.

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