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A novel thermophilic lysozyme from bacteriophage φIN93

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ABSTRACT

The lysozyme of bacteriophage φIN93 was purified to apparent homogeneity with Carboxymethyl Sepharose and Hydroxyapatie columns from lysates of the phage grown on *Thermus aquaticus* TZ2. The enzyme is a single polypeptide chain with a molecular weight of 33,000. From the determined N-terminal amio acids of the enzyme, the locus of the gene was specified on a φIN93 genome. The enzyme was not similar to egg white lysozyme, T4 phage lysozyme, or lambda phage lysozyme. The enzyme, φIN93 lysozyme, was found to be a novel type of thermophilic lysozyme, which lyses specifically *Thermus* sp. cells, and exhibited conspicuous thermal stability at 95 °C for 1 h in the presence of β-mercaptoethanol.

We induced a bacteriophage φIN93 from a thermophilic bacterium *Thermus aquaticus* TZ2 growing at 70 °C, which was isolated from hotspring soil [1]. φIN93 is a lysogenic phage, but under lytic cycle, φIN93 lyses the host *Thermus aquaticus* TZ2. Thus, we deduced that φIN93 may have a thermophilic lysozyme. On a thermophilic lysozyme reacting above 70 °C, there has yet been no report.

In this paper, we describe the purification, characterization, and locus of the gene of the thermophilic lysozyme which φIN93 has.

Materials and methods

Bacteria and phage. Thermus aquaticus TZ2 was used as the host cell and substrate of lysozyme. The phage was φIN93. *Thermus thermophilus* ATCC 27634 (HB8), *Thermus thermophilus* DSM 7039 HB27, *Thermus aquaticus* DSM625^T (YT1), *Thermus filiformis* DSM4687^T, *Escherichia coli* IFO3972, *Pseudomonas aeruginosa* IFO13275, *Bacillus subtilis* IFO3134, *Staphylococcus aureus* IFO13276, *Salmonella typhimirium* IFO152, and *Micrococcus lysodeikticus* (ATCC 4698) were purchased from respective institutions.

Media. The A-2 medium used contained 0.1% tryptone, 0.1% yeast extract, and Castenholtz basal salts (pH 7.0). Castenholtz basal salts are composed of 0.1 g of nitrilotriacetic acid, 0.06 g of $CaSO_4{\cdot}2H_2O$, 0.1 g of $MgSO_4{\cdot}7H_2O$, 0.008 mg of NaCl, 3.16 mg of $MnSO_4{\cdot}5H_2O$, 0.5 mg of $ZnSO_4{\cdot}7H_2O$, 0.5 mg of H_3BO_3, 0.016 mg of $CuSO_4$, 0.103 g of KNO_3, 0.689 g of $NaNO_3$, 0.111 g of Na2HPO$_4$, 0.42 mg/L of $FeCl_3{\cdot}6H_2O$, 0.025 mg of $Na_2MoO_4{\cdot}2H_2O$, 0.046 mg of $CoCl_2{\cdot}6H_2O$, and 0.5 μg of H_2SO_4 in a final volume of l liter.

Reagents. Carboxymethyl Sepharose™ Fast Flow was obtained from Amersham Biosciences Co. Macro-Prep Ceramic Hydroxyapatite TYPE1 was from BioRad Co.

Preparation of phage lysate. Thermus aquaticus TZ2 was grown overnight at 70 °C in 5 ml of A-2 medium. To amplify φIN93, 75 ml of the A-2 medium in a 300 ml flask was inoculated with 0.15 ml of the overnight culture of *Thermus aquaticus* TZ2 and incubated while being shaken at 220 rpm at 70 °C. Eight flasks of the above culture were prepared and the total culture volume was 600 ml. When the bacteria reached 10^8 cfu/ml, phage were added (10^6 pfu/ml, final concentration), and incubation was continued by shaking at 220 rpm until complete lysis occurred (after 2.5 h). Cell debris in the resulting φIN93 lysate was removed by centrifugation with a swing centrifuge (Model CT6E Hitachi, Ltd.) at 3500 rpm for 20 min and the supernatant of the lysate was recovered.

Purification of phage lysozyme. The φIN93 lysozyme obtained was purified according to the purification methods of lambda phage lysozyme and T4 phage lysozyme [2,3]. All the manipulations in the following procedure were performed at room temperature.

Step1: Filtration. The above lysate was applied to an Amicon ultra 100,000 MWCO filter and centrifuged at 3500 rpm for 20 min. To concentrate the enzyme, this process was carried out several times. After that, 10 mM sodium phosphate pH 6.0 was added to the supernatant and centrifuged at 3500 rpm for 20 min. This process was carried out two times for buffer exchange. After centrifugation, the supernatant was prepared in a final volume of 5 ml to obtain Fraction I.

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Step2: Carboxymethyl Sepharose™ Fast Flow Column Chromatography. A column of Carboxymethyl Sepharose™ Fast Flow (1.5 cm×5 cm) was equilibrated with 10 mM sodium phosphate pH 6.0. Fraction I was applied to the column at a flow rate of 1.0 ml/h. The column was washed with 25 ml of 10 mM sodium phosphate pH 6.0 and stepwise elution from 0.1 M NaC to 0.5 M NaCl was applied at a flow rate of 1.0 ml/min, 2 ml fractions being collected. Each fraction was applied to the Amicon ultra 10,000 MWCO filter and centrifuged at 3500 rpm for 20 min to remove NaCl. To the supernatant of each fraction, 10 mM sodium phosphate pH 7.0 was added and centrifuged under the above conditions. This process was carried out two times for buffer exchange. The final volume of each fraction prepared was 2 ml. The fractions containing enzyme were accumulated to form Fraction II.

Step3: Hydroxyapatite Column Chromatography. A column of Macro-Prep Ceramic Hydroxyapatite TYPE1 (1.0 cm×1.3 cm) was equilibrated with 10 mM sodium phosphate pH 7.0. Fraction II was applied to the column at a flow rate of 1.0 ml/h. The column was washed with 10 ml of 10 mM sodium phosphate pH 7.0 and stepwise elution from 0.1 M NaCl to 0.5 M NaCl in10 mM sodium phosphate pH 7.0 followed at 1.0 ml/min, 1 ml fractions being collected. Each fraction was applied to the Amicon ultra 10,000 MWCO filter and centrifuged at 3500 rpm for 20 min. The following process was carried out in the same way as Step 2. The final volume of each fraction prepared was 1 ml. The fractions containing enzyme were accumulated to form Fraction III.

Step4: Second Hydroxyapatite Column Chromatography. The same column of Macro-Prep Ceramic Hydroxyapatite TYPE1 as in Step 3 was equilibrated with 10 mM sodium phosphate pH 7.0. Fraction III was applied to the column at a flow rate of 1.0 ml/h. The column was washed with 10 ml of 10 mM sodium phosphate pH 7.0 and stepwise elution from 0.1 M NaCl to 0.5 M NaCl in 10 mM sodium phosphate pH 7.0 followed at 1.0 ml/min, 0.5 ml fractions being collected, different from Step 3. Each fraction was applied to the Amicon ultra 10,000 MWCO filter and centrifuged at 3500 rpm for 20 min. The following process was carried out in the same way as in Step 2. The final volume of each fraction prepared was 0.5 ml. The fractions containing enzyme were accumulated to form FractionIV.

Assay of lysozyme. The activity of the enzyme was assayed by observing its ability to decrease the absorbance at 540 nm caused by the turbidity of chloroform-treated *Thermus aquaticus* TZ as the substrate, according to the method of Lindsay W. Black [2]. The bacterial substrate was prepared from cells growing exponentially at 70 °C in 1 liter of A-2 medium. The cells were harvested by centrifugation at 3500 rpm for 20 min when their concentration was 10^8 cfu/ml and then suspended in 20 ml of 10 mM sodium phosphate pH 7.0. An equal volume (20 ml) of chloroform was added to the suspension and then shaken several times. By centrifugation (for 20 min, at 3500 rpm), the cells separated in the middle layer were harvested and then lyophilized to remove chloroform. The lyophilized cell powder was suspended in dilution solvent (10 mM sodium phosphate, pH 7.0) so as to be adjusted to OD 1.2 in absorbance (at 540 nm). Enzyme activity was measured with the cell suspension as the substrate. Then 0.01 ml of the enzyme fractionated in each purification step was added to a tube which contained 0.5 ml of the above substrate and incubated at 70 °C for 2 min. The contents were transferred to a cuvette, and the absorbance at 540 nm was determined. One unit of the enzyme was defined as that amount which will cause an absorbance of decrement 0.1.

Optimum temperature and pH. Enzyme activity at various temperatures from 37 °C to 150 °C was measured. At various pH values, it was measured in 10 mM citrate–phosphate buffer pH 5, 10 mM sodium phosphate buffer from pH 6 to pH 8, and 10 mM glycin–NaOH buffer from pH 9 to pH 11.

Thermal stability. The enzyme was incubated at 95 °C for 1 h or 6 h in 10 mM sodium phosphate buffer pH 7.0 with a final concentration of 25 mM of β-mercaptoethanol or without β-mercaptoethanol, and then lysozyme activity was measured. Relative activity was calculated as the ratio of the enzyme activity incubated at 95 °C, to that with no incubation.

Substrate specificity. The substrate specificity of the enzyme was examined by comparing the enzyme activity to *Thermus aquaticus* TZ2 as the substrate with enzyme activity to the following bacteria: thermophilic bacteria (*Thermus thermophilus* HB8, *Thermus thermophilus* HB27, *Thermus aquaticus* YT1, and *Thermus filiformis*) and mesophilic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimirium*, and *Micrococcus lysodeikticus* (ATCC 4698)).

SDS–polyacrylamide gel electrophoresis and N-terminal amino acids analysis. Purified enzyme (FractionIV), boiled at 95 °C for 5 min in sodium dodecylsulfate (SDS) and β-mercaptoethanol, was separated by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto a polyvinilidene difluoride (PVDF) membrane by using Horize blott AE-6677 (ATTO Co). Blotting was performed at the rate of 2 mA/cm^2 for 30 min. The protein band was stained with Coomassie Brilliant Blue R-250. The NH_2-terminal amino acid sequences of the purified enzyme were determined with PROCISE-cLC (Applied Biosystems Co).

Other assays and measurements. Protein was determined from the absorbance at 280 nm according to the following formula:

$$\text{Protein concentration (mg/ml)} = 1.115 \times \text{Abs}_2 80$$

Gene analysis. The locus of the lysozyme gene on the φIN93 genome was determined from the sequence of N-terminal amino acids by using the computer software GENETYX WIN (GENETYX Co). The BLAST and PSI-BLAST were used to detect the homologs of the lysozyme in the DNA and protein database.

Results and discussion

Enzyme purification

In Step 1, enzyme activity was detected only in the supernatant of phage lysate centrifuged by the Amicon ultra 100,000 MWCO filter. In the result of Carboxymethyl Sepharose™ Fast Flow Column Chromatography, enzyme activity was detected in the fraction of 0.1 M and 0.2 M NaCl (Fraction II). In the Hydroxyapatite Column Chromatography following the above chromatography, enzyme activity was detected in the fraction of 0.3 M NaCl (Fraction III). In a second Hydroxyapatite Column Chromatography, enzyme activity was also detected in the fraction of 0.3 M NaCl. A summary of the purification procedure, in terms of the recovery and specific activity at each step, is given in Table 1. The purified enzyme had a specific activity of 31,045 U/mg and was purified 2600 times more than the original lysate. The final recovery of activity was about 11%. In the result of electrophoresis of the purified enzyme in SDS–polyacrylamide gel, only one component of Mw. 33,000 was detectable.

Optimum temperature, pH, and thermal stability

A phage lysozyme reacts when a phage infects the host cell and bursts out after proliferating in the host cell; thus, φIN93 lysozyme is considered to react under the same condition as when *Thermus aquaticus* TZ2 can grow and be infected by φIN93. As shown in Fig. 1(A), the optimum temperature for enzyme activity was between 60 and 120 °C. Enzyme activity was stable at these temperatures. From the result, φIN93 lysozyme was found to be a thermophilic enzyme, different from the other lysozymes, T4

Table 1
Purification of φIN93 lysozyme

Fraction and step	Volume (ml)	Protein	φIN93 lysozyme		Yield (%)	Fold
		Total (mg)	Total (U)	Specific activity (U/mg)		
Lysate	600		–	–	–	
Filtration supernatant	5.0	167.5566	1974	12	100	1
CM Sepharose	4.0	4.4600	1581	354	80	30
Hydroxyapatite (1)	2.0	0.1293	685	5296	35	449
Hydroxyapatite (2)	0.5	0.0072	225	31,045	11	2635

One unit of the enzyme is defined as that amount which will cause an absorbance decrement of 0.1.

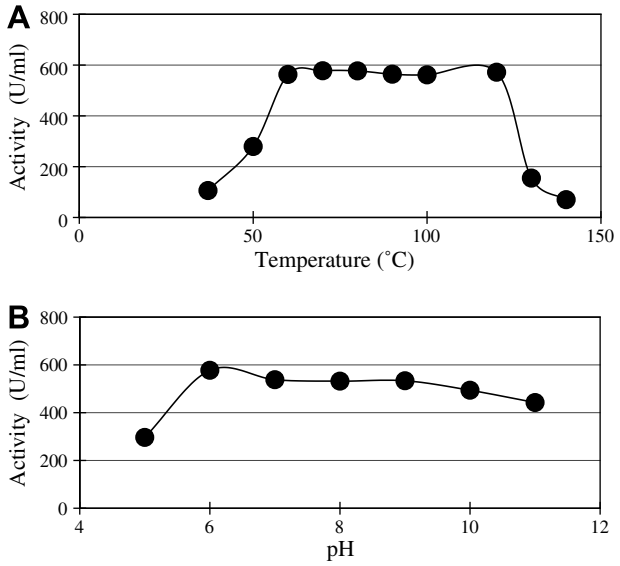

Fig. 1. Optimum conditions of φIN93 lysozyme. (A) Temperature–activity curve and (B) pH–activity curve. The enzyme assay was carried out as described in the text.

phage tail lysozyme, e lysozyme [4,5] lambda phage lysozyme [6,7], and egg white lysozyme [8,9] that had previously been studied. As shown in Fig. 1(B), the optimum pH was stable between pH 6 and pH 10.

The stability of the enzyme at elevated temperature is shown in Fig. 2. After incubation at 95 °C for 1 h with β-mercaptoethanol, 93.8% of enzyme activity remained, while without β-mercaptoethanol, the residual enzyme activity was only 38.6%. Although we do not have any data elucidating the mechanism of thermal stability, the Cystein residue (at position of 112) of φIN93 lysozyme seemed to be protected from oxidation by β-mercaptoethanol. The enzyme activity, however, was not enhanced by β-mercaptoethanol (data not shown).

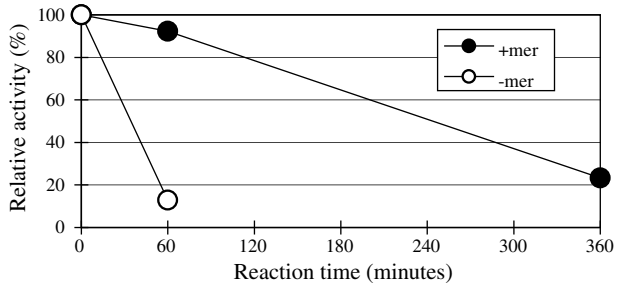

Fig. 2. Thermal stability of φIN93 lysozyme. The enzyme was incubated at 95 °C in 10 mM phosphate buffer, pH 7.0, for 0–6 h. After incubation, the solution was cooled to room temperature and the residual activity was measured. Activity is expressed as the percentage of activity under no incubation.

Substrate specificity

From the result of comparing the relative activities of thermophilic *Thermus* sp. and of mesophilic bacteria, it was found that *Thermus thermophilus* HB8 (especially of high value: 79%), *Thermus thermophilus* HB27, *Thermus aquaticus* YT1, and *Thermus filiformis* had high activities from 42% to 79% (*Thermus aquaticus* TZ2 as the substrate: 100%), while mesophilic bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimirium*, *and Micrococcus lysodeikticus* showed no activity (see Table 2).

The difference in activity between *Thermus* sp. and mesophilic bacteria may be due to the structure of peptidglycan. The peptidglycan of *Thermus thermophilus* HB8 has Ornitin residue different from diaminopiperic acid of *Escherichia coli* and Lysine of *Staphylococcus aureus* [10]. The φIN93 lysozyme obtained may recognize not only GlcNac–MurNac but also the amino acid sequence of the peptidglycan.

Thermus aquaticus TZ2 had also Ornitin residue from the analysis of the cell wall [1]. The nucleotide sequences of 16SrRNA between *Thermus aquaticus* TZ2 and *Thermus thermophilus* HB8 were completely the same. Thus, the cause of the high relative activity to *Thermus thermophilus* HB8 as the substrate was considered to be *Thermus aquaticus* TZ2 and *Thermus thermophilus* HB8 being of the same species. From these results, φIN93 lysozyme was found to be a novel type which catalyzes the hydrolysis of peptideglycan from *Thermus* sp. being of same species between

Gene analysis of lysozyme gene

The NH_2-terminal amino acid sequence of φIN93 lysozyme was determined (10 residues, Met-Ser-Val-Arg-Ile-Thr-Asn-Phe-Gly-Leu). The locus of the lysozyme gene was determined on the φIN93 genome from the data on the NH_2-terminal amino acid sequence. The lysozyme gene was ORF24 (position: 11924–12841) which starts from ATG codon and has 918 bp. The sequence of the lysozyme gene was deposited in the Genbank/EMBL/DDBJ database under Accession No. AB063393.

The molecular weight (33,141), predicted from the amino acid sequence of ORF24, was almost the same as that from the result

Table 2
Substrate specificity of φIN93 lysozyme

Cell treated with chloroform		Relative activity (%)
Thermus aquaticus TZ2		100
Thermus aquaticus YT1	DSM625T	42
Thermus filiformis	DSM4687T	76
Thermus thermophilus HB8	ATCC27634	79
Thermus thermophilus HB27	DSM7039	61
Bacillus subtilis	IFO3134	14
Escherichia coli	IFO3972	0
Pseudomonas aeruginosa	IFO13275	0
Salmonella typhimirium	IFO152	0
Staphyro coccus aureus	IFO13276	0

Relative activities are expressed as the percentage of activity to *Thermus aquaticus* TZ2.

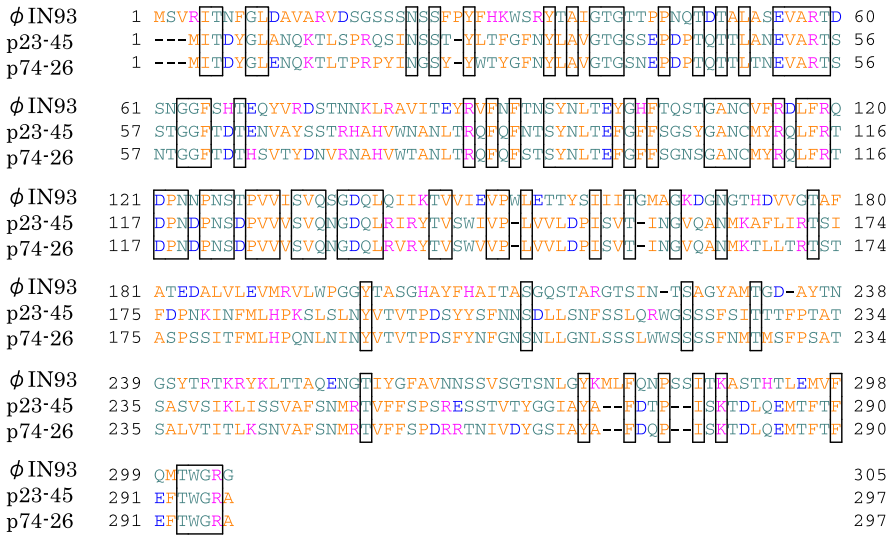

Fig. 3. Multialignment of φIN93 lysozyme, *Thermus* phage p23-45 and p74-26 hypothetical proteins. Identical amino acid residues are boxed. The yellow type indicates the hydrophobic residues, the blue type indicates the acidic residues, the pink type indicates the basic residues, and the green type indicates the neutral residues. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of SDS–PAGE. In Step 1 of the enzyme purification procedure, however, enzyme activity was detected in the supernatant fractionated with the Amicon ultra 100,000 MWCO filter. From these results, φIN93 lysozyme was capable of forming a functional complex with other proteins as in the case of T4 phage tail lysozyme [4,11–13].

In the results of homology analysis with the protein database, φIN93 lysozyme was similar to hypothetical protein of *Thermus* phage P74-26 and *Thermus phage* P23-45 (297 amino acids, homogeneity: 31.5%), but not to other lysozymes, T4 phage tail lysozyme, e lysozyme [4,5], lambda phage lysozyme [6,7], and egg white lysozyme [8,9] (Fig. 3). These hypothetical proteins of *Thermus* phage P74-26 and *Thermus phage* P23-45 may also have lysozyme activity specific to *Thermus* sp. Furthermore, the amino acid sequences of ORF23 and ORF25 next to ORF24 on the φIN93 genome were also similar to hypothetical proteins of *Thermus* phage P74-26 and *Thermus phage* P23-45. These proteins may cooperatively form a complex with the purified lysozyme.

On the active site, T4 phage lysozyme, lambda phage lysozyme, and egg white lysozyme have already been clarified, but not φIN93 lysozyme. Further work is needed to clarify the active site, each role of the proteins predicted to form a complex with lysozyme, the cause of substrate specificity, and the mechanism of thermal stability.

If thermophilic lysozyme can be produced commercially, it will be used for many kinds of application such as medical supplies, cosmetics, agrochemicals, preservatives, and filters of air cleaners.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.101.

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