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Cloning and Expression of a Chitinase Gene from *Thermoactinomyces vulgaris* KFB-C100

YOON, HO-GEUN, HEE-YUN KIM[1], YOUNG-HEE LIM*, AND HONG-YON CHO

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
[1]Department of Food Additives, Korea Food and Drug Administration, Seoul 122-202, Korea

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Abstract We have found that *Thermoactinomyces vulgaris* KFB-C100 produces a chitinase. The optimum temperature and pH of the enzyme activity were 55°C and 6.5. The enzyme was stable after heat treatment at 80°C for 30 min and stable in acidic and basic conditions (pH 6.0~11.0). The thermostable endo-chitinase from *Thermoactinomyces vulgaris* KFB-C100 was cloned into the plasmid pBR322 by using *E. coli* DH5α as a host strain. The positive clone carrying a recombinant plasmid (pKCHI23) with a 4.1-kb fragment containing the chitinase gene was found. The recombinant plasmid was analyzed to determine the essential region for chitinase activity and obtained a 2.3-kb fragment, which was subcloned into pTrc99A using the *Pst*I and *Sal*I sites to construct pTrc99A/pKCHI23-3. The resulting plasmid exerted high chitinase activity upon transformation of *E. coli* XL1-Blue cells. Chitinase was overproduced 14 times more in the clone cells than in the wild-type cells and the enzyme was purified to homogeneity. The purified enzyme showed the similar properties as the native chitinase from *T. vulgaris* in terms of molecular weight and substrate specificity. The catalytic action of the cloned enzyme was an endo type, producing chitobiose as a major reaction product.

Key words: Chitinase, cloning, expression, thermostable enzyme

Chitin-based products have been used widely in various fields and new uses have been developed recently in the field of human nutrition, medical aids, and pharmaceuticals [9]. Chitinase hydrolyzes polymers of (1→4)-β-D-linked *N*-acetylglucosamine (GlcNAc) residues to chitooligomers. A wide range of bacteria, fungi, plants, insects, fishes, and some other animals produce the enzyme [8, 14, 16, 20].

Several chemical methods for hydrolysis of chitin have been reported [2, 3]. Complete chemical hydrolysis of chitin requires a harsh treatment with a high concentration of acid, which may cause loss of valuable chitooligomers. Furthermore, this acid hydrolysis is not specific, which means that oligosaccharides from other polysaccharide derivatives, as well as chitin, are produced by this method. On the other hand, enzymatic hydrolysis with chitinases has been performed in mild conditions and showed specificity on chitin and its derivatives. Several chitinases have been used commercially, although their reaction mechanisms and active structures have not been fully characterized [1, 15, 22]. A large-scale hydrolysis of chitin in industry has been narrowly restricted because of the high cost of the enzymes. Recently, several studies on the cloning of the chitinase gene have been reported to increase the productivity of the enzyme, resulting in reduction of product cost [4, 5, 19, 21].

We found that a thermophilic bacterium *Thermoactinomyces vulgaris* produced a thermostable chitinase. We describe here the cloning and overexpression of the endo-chitinase gene of *T. vulgaris* KFB-C100 in the *E. coli* XL1-Blue cells. In addition, this paper describes the purification and characterization of thermostable chitinase from the recombinant *E. coli* cells.

MATERIALS AND METHODS

Materials

Colloidal chitin was prepared by Lockwood's method [6]. Chitin, *N*-acetyl-D-glucosamine, Lysozyme, and RNase were from Sigma Co. (St. Louis, U.S.A.). Chitobiose, chitotriose, and the other chitooligosaccharides were purchased from Seikagaku Co. (Tokyo, Japan). Vectors used (pBR322, pKK223-2, and pTrc99A) were obtained from Pharmacia (Uppsala, Sweden). Restriction enzymes and T4 DNA ligase were from Promega Co. (Madison, U.S.A). All other reagents were of analytical grade.

*Corresponding author
Phone: 82-2-923-8732; Fax: 82-2-923-8733;
E-mail: hy07@kuccnx.korea.ac.kr

Microorganism and Cultivation

A strain of *T. vulgaris* KFB-C100, which was selected as the most potent thermostable chitinase producer from the stock strains in our laboratory, was used for the production of the enzyme. The culture medium consisted of 1.2% colloidal chitin, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.1% yeast extract (pH 6.5). The microorganism was precultured in the medium containing 1.2% glycerol instead of colloidal chitin. For the production of the enzyme, the precultured microorganism (1%, v/v) was added in a 500-ml baffle flask with 100 ml of the culture medium and cultivated at 55°C for 40 h with agitation at 150 rpm.

Isolation and Cloning of DNA

Chromosomal DNA was prepared from *T. vulgaris* KFB-C100 by Marmur's method [12]. The DNA was partially digested with *Bam*HI and electrophoresed on a 1.0% agarose gel. Fragments with the size of 4~10 kb were collected using a Prep-A Gene® DNA Purification kit (Bio-Rad, Hercules, U.S.A.). pBR322 plasmid was cleaved at the *Bam*HI site and treated with calf intestinal alkaline phosphatase (Promega Co., Madison, U.S.A). The *Bam*HI fragments from chromosomal DNA were ligated into the dephosphorylated *Bam*HI site of pBR322. The overall cloning strategy is summarized in Fig. 1. The ligation mixture was inserted into *E. coli* DH5α, and the transformants were screened on Luria-Bertani's (LB) agar plates containing chitin (1.2%) and ampicillin (50 mM). Colonies forming a clear zone around them on LB-chitin agar plates were transferred to filter paper (Whatman No. 2). The filter paper was soaked in a reaction solution containing 30 μM of 4-methylumbelliferyl-β-D-*N,N'*-diacetyl chitobioside (4-MUF(GlcNAc)$_2$) in 0.1 M phosphate

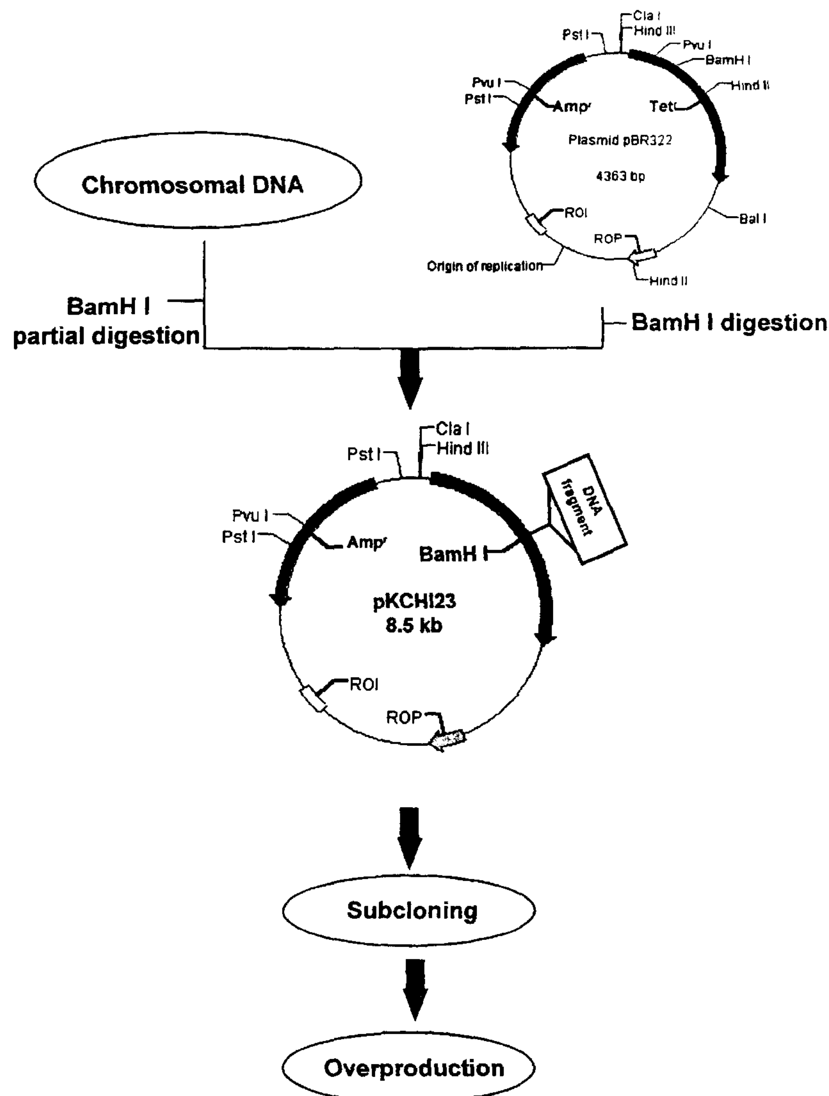

Fig. 1. Cloning strategy of the thermostable chitinase gene from *T. vulgaris* KFB-C100.

buffer (pH 7.4) and incubated at 37°C for 10 min. After adding the saturated sodium bicarbonate solution to the reaction solution, the filter paper was irradiated with UV light (366 nm) and colonies forming a light blue fluorescence were selected as the positive clones, indicating clones carrying pBR322 with the chitinase gene [18]. The plasmid isolated from a positive transformant was designated as pKCHI23, which contained a 4.1-kb fragment of the *T. vulgaris* genomic DNA.

Analysis of Recombinant Plasmid, pKCHI23, Carrying the Chitinase Gene

The recombinant plasmid was digested with *Bam*HI and the insert DNA was isolated by agarose gel electrophoresis. The isolated insert DNA was used for restriction mapping and subcloning. Restriction maps were constructed by using single and multiple digestions of recombinant plasmids. Restriction enzymes were used according to the manufacturer's specifications. Various lengths of the inserted 4.1-kb region of pKCHI23 was unidirectionally deleted from each side. The deletion mutants of pKCHI23 obtained were introduced into *E. coli* XL1-Blue. The chitinase activity of each transformant was assayed. Plasmid DNAs from recombinants were prepared by alkaline lysis procedure [17].

Construction of the Overexpression Plasmid

A 2.3-kb gene fragment found to be necessary for the enzyme production was ligated into the expression vectors, pKK223-2 and pTrc99A, which were digested with *Pst*I and *Sal*I. The resultant plasmids exhibited chitinase activity and were named pTrc99A/pKCHI23-3 and pKK223-2/pKCHI23-3, respectively.

Enzyme and Protein Assays

The reaction mixture containing 250 μl of 1.0% chitin, 50 μl of 1.0 M potassium phosphate buffer (pH 6.5), and the enzyme solution in a final volume of 1 ml was incubated at 55°C for 30 min in a shaking water bath. The reaction was stopped by heating at 100°C for 10 min, followed by centrifugation. The amount of reducing sugars in the supernatant was determined by the dinitrosalicylic acid (DNS) method with modification of the method of Miller [13]. One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per hour. *N*-acetyl-D-glucosamine was used as a standard. Protein concentration was determined by the Lowry method [11] with bovine serum albumin as a standard.

Purification of Chitinase from *E. coil* XL1-Blue pTrc99A/pKCHI23-3 cells

Transformants were grown in LB broth containing 50 mM of ampicillin with isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C with shaking. After cultivation, the cells were removed by centrifugation at 14,000×g for 30 min. 0.1 mM phenylmethylsulfonyl fluoride and 0.05 mM 1-tosylamido-2-phenylethylchloromethyl ketone were added to the supernatant and the supernatant was used as the crude enzyme extract solution.

Ammonium sulfate fractionation. Powdered ammonium sulfate was added to the crude enzyme extract solution to obtain 30% saturation. After 30 min the supernatant was collected by centrifugation at 14,000×g for 10 min and additional ammonium sulfate was added to 70% saturation. After standing for 1 h, the precipitate was collected by centrifugation and dissolved with a minimum volume of the buffer (10 mM phosphate). The enzyme solution was dialyzed against the phosphate buffer.

Heat treatment. After the ammonium sulfate was removed, the solution was incubated at 70°C for 30 min. The denatured proteins were removed by centrifugation.

Butyl-Toyopearl column chromatography. The enzyme solution saturated with ammonium sulfate (30%) was applied to a Butyl-Toyopearl 650 M column (Tosoh, Tokyo, Japan, 1.6×30 cm) equilibrated with the buffer containing ammonium sulfate (30% saturated) and washed with the same buffer. A linear gradient was performed with the buffer supplemented with ammonium sulfate by decreasing the concentration from 30 to 0% saturation. The active fractions were pooled and concentrated by ultrafiltration.

TSK-Gel filtration. The enzyme solution was subjected to gel filtration with a TSK-Gel HW-55F column (1.5×70 cm, Tosoh, Tokyo, Japan) equilibrated with the buffer containing 50 mM KCl. The enzyme was eluted with the same buffer at a flow rate of 10 ml/h. The active fractions were pooled, dialyzed, concentrated by ultrafiltration, and stored at -70°C.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was performed in 10% polyacrylamide gel containing 0.1% SDS by Laemmli's method [10].

Measurement of Molecular Weight

The molecular weight of the enzyme was determined by TSK-Gel permeation chromatography. Cytochrome C (12.5 kDa), chymotrypsinogen (25 kDa), hen egg albumin (45 kDa), bovine serum albumin (68 kDa), aldolase (158 kDa), catalase (240 kDa), and ferritin (450 kDa) from Boehringer Mannheim (Mannheim, Germany) were used as standards.

Analysis of the Products of the Enzymatic Hydrolysis

The reaction mixture, 700 μl of enzyme solution (5 mg/ml), 250 μl of 20% colloidal chitin, and 50 μl of 1.0 M potassium phosphate buffer (pH 7.0) containing 0.01% NaN_3, was incubated at 55°C for 12 h. The reaction was

stopped by heating at 100°C for 10 min, followed by centrifugation. The products in the supernatant were analyzed by HPLC with a YMC-pack polyamine II column, and eluted with an acetonitrile-water mixture (75:25). The chitooligosaccharide eluted was detected with a refractive index (RI) detector. *N*-acetyl-D-glucosamine, chitobiose, chitotriose, chitotetrose, chitopentose, and chitohexose were used as authentic standards.

RESULTS AND DISCUSSION

Properties of Chitinase from *T. vulgaris*

Extracellular and intracellular chitinase activities were detected after *T. vulgaris* KFB-C100 was cultivated at 55°C. As shown in Fig. 2, the cell growth reached its maximum after 24 h of growth. The enzyme activity appeared in the exponential phase of cell growth and increased rapidly to the maximum value, about 5 units/ml in extracellular medium, after 48 h. However, intracellular activity was not found until 60 h. Chitinase was purified and its properties were examined. The molecular weight of the enzyme was 59 kDa and the enzyme consisted of a monomer (data not shown).

Effects of Temperature and pH on Enzyme Activity and Stability

The optimal temperature for chitinase activity was examined. The mixtures of the enzyme and colloidal chitin in 20 mM potassium phosphate buffer, pH 6.5, were incubated at various temperatures for 30 min. The enzyme was the most active at 55°C under the standard assay condition (data not shown). The optimal pH for chitinase activity was examined with acetate buffer (pH 3.0 to 6.5), potassium phosphate buffer (pH 6.5 to 8.5), and glycine buffer (pH 8.5 to 11.0). The optimal pH was 6.5 (data not shown).

The effect of temperature on enzyme stability was examined at pH 6.5. After treatment of the enzyme at 80°C for 10 min the remaining activity was 75% and after 30 min, 50%. 90% of initial activity was retained after incubation at 60°C for 30 min. The enzyme activity was stable up to 60°C (Fig. 3A). In order to determine the pH stability of the enzyme, chitinase was incubated at 55°C for 30 min at various pHs. After adjusting the enzyme solution to pH 6.5, the remaining activity was determined. The remaining activity was over 80% at pH 6.0

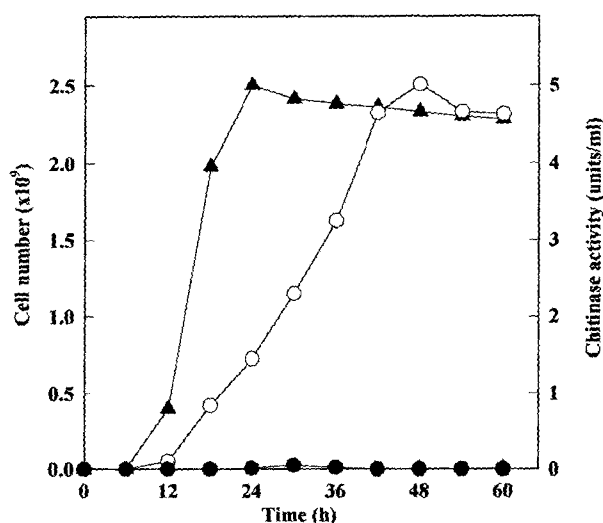

Fig. 2. Activities of extracellular (○-○) and intracellular (●-●) chitinase during the growth of *T. vulgaris* KFB-C100 (▲-▲).
After cells were cultivated under the optimum culture conditions for the various times indicated, the culture mixture was collected and the enzyme assay was performed as described in MATERIALS AND METHODS.

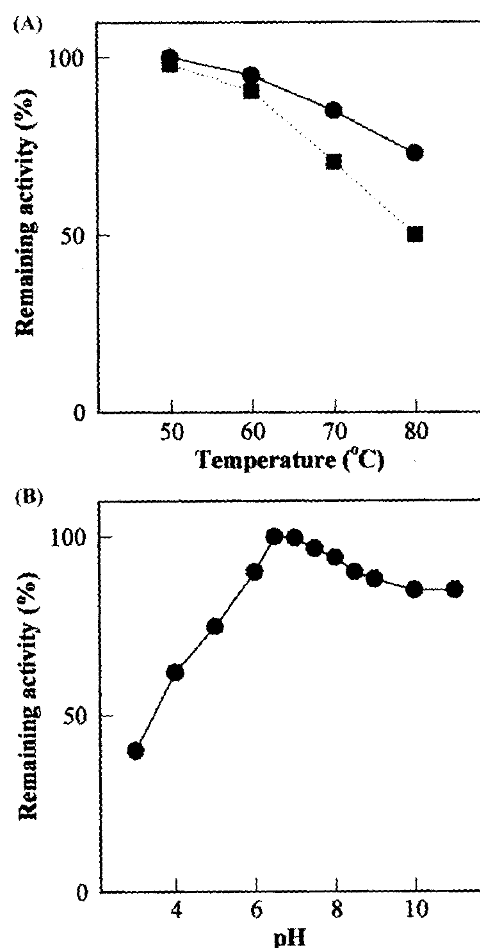

Fig. 3. Effects of temperature and pH on the enzyme stability.
(A) After the enzyme was incubated at 50, 60, 70, and 80°C for 10 min (●) and 30 min (○), remaining activities were measured for determining the thermostability of the enzyme. (B) The purified enzyme was incubated in the various pH range buffers mentioned in Fig. 2 at 55°C for 30 min. After adjusting the reaction solutions to pH 6.5, the remaining activities were measured to determine the pH stability of the enzyme.

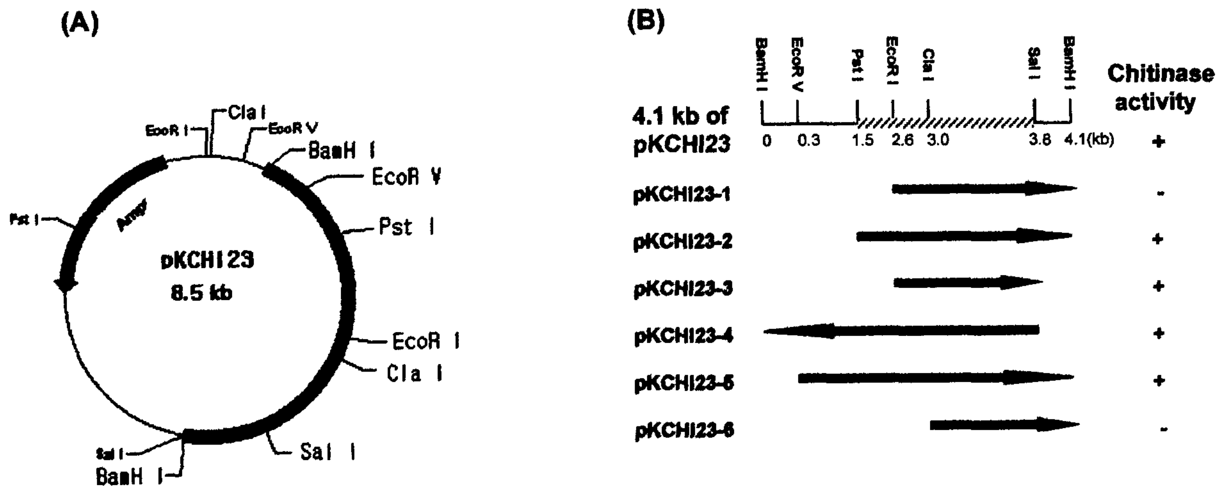

Fig. 4. Restriction map (A) and deletion analysis (B) of pKCHI23.

+, Chitinase activity detected; -, Chitinase activity not detected. The oblique line indicates the region necessary for the expression of chitinase. The arrows indicate the direction of deletion.

to 11.0 (Fig. 3B). These results indicate that the enzyme is relatively stable in both acidic and basic conditions.

Cloning of the *T. vulgaris* Chitinase Gene

The recombinant *E. coli* DH5α containing the library clones of *T. vulgaris* KFB-C100 genomic DNA was screened for the expression of the chitinase gene. We selected the transformant possessing the chitinase gene by exposing the cell to UV light (366 nm) after soaking with 4-methyumbelliferyl-β-D-*N,N'*-diacetyl chitobioside. Recombinant *E. coli* cells producing chitinase show a light blue fluorescence, 4-methylumbelliferone, formed by the action of chitinase. Among about 10,000 ampicillin-resistant and tetracycline-sensitive colonies, only one clone showed a blue fluorescence, derived from chromogenic dye (4-MUF$(GlcNAc)_2$), indicating the clone contained a chitinase gene. *E. coli* XL1-Blue cell and *E. coli* XL1-Blue/pBR322 cell did not show any blue fluorescence. Only *E. coli* XL1-Blue/pKCHI23 cell showed a blue fluorescence, which suggested that the cloned fragment was derived from the chitinase gene of *T. vulgaris* KFB-C100 (data not shown). The insert size of the plasmid isolated from the positive transformant (pKCHI23) was 4.1 kb. The recombinant plasmid was digested with *Bam*HI and the insert DNA of 4.1 kb was isolated by agarose gel electrophoresis. The isolated insert DNA was used for subcloning and restriction mapping.

The restriction map and deletion analyses of 4.1-kb insert of pKCHI23 are shown in Fig. 4. To determine the location of the chitinase gene in the 4.1-kb insert DNA, a series of deletion mutants of pKCHI23 was constructed and the chitinase activity assayed. Deletions of a 1.5-kb region from left to right and of a 1.3-kb region from right to left did not affect the expression of chitinase activity. It was found that the 2.3-kb *Pst*I-*Sal*I fragment was the region necessary for the production of chitinase.

Construction of an Over-Producer of Chitinase

To increase the enzyme productivity, the 2.3-kb gene fragment, pKCHI23-3, was ligated into pTrc99A and pKK223-2 expression vectors and the over-producers, pTrc99A/pKCHI23-3 and pKK223-2/pKCHI23-3, were constructed. The specific activities of the enzyme from these over-producers were compared with that of the enzyme from the wild-type *T. vulgaris*. The specific activities of

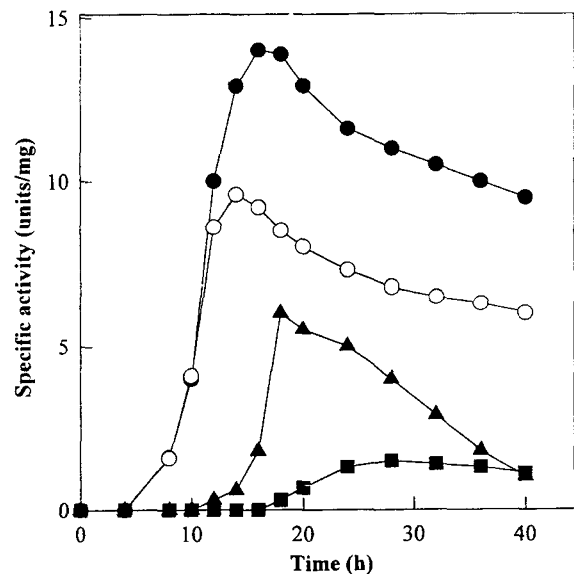

Fig. 5. Overexpression of chitinase using various expression vectors in *E. coli* XL1-Blue.

Specific activities of extracellular fractions from recombinant cells: pUC18/pKCHI23-3 (▲), pKK223-2/pKCHI23-3 (○), pTrc99A/pKCHI23-3 (●), and from *T. vulgaris* (■).

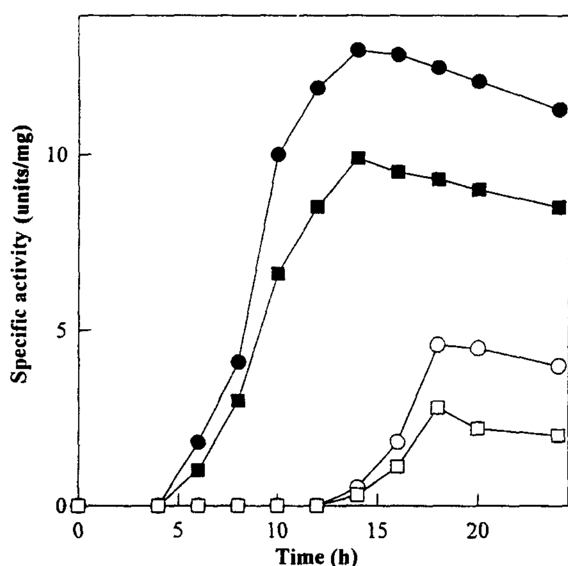

Fig. 6. Production of chitinase from *E. coli* XL1-Blue pTrc99A/pKCHI23-3 in various media.

Specific activity of extracellular fractions from recombinant cells in LB medium (□): LB medium containing 1.2% of colloidal chitin and 120 mM of IPTG (●); 120 mM of IPTG (■); 1.2% of colloidal chitin (○).

the enzymes produced by *E. coli* XL1-Blue pTrc99A/pKCHI23-3, *E. coli* XL1-Blue pKK223-2/pKCHI23-3, and *E. coli* XL1-Blue pUC18/pKCHI23-3 were about 14-, 10-, and 6-fold higher, respectively, than that of the enzyme from *T. vulgaris* (Fig. 5). To investigate the effect of IPTG and chitin on the production of chitinase, the recombinant cells were cultivated in various culture media. As shown in Fig. 6, the enzyme production was increased in the medium containing chitin compared with that in the medium without chitin. When IPTG was added to the LB medium, the specific activity of the enzyme was increased about four times. The result shows that the expression of the chitinase gene from those constructs is induced by IPTG.

Table 1. Purification of chitinase from *E. coli* XL1-Blue pTrc99A/pKCHI23-3.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yields (%)	Purification folds
Crude enzyme	380	5,560	14.6	100	-
Ammonium sulfate fractionation (30~70%)	127	3,940	31.0	71	2.1
Heat treatment*	24.8	1,969	81.8	51	5.4
Butyl-Toyopearl chromatography	20.4	534	262	10	18
TSK-Gel HW-55F gel filtration	1.12	356	318	6.4	22

*Heat treatment was performed at 70°C for 30 min.

Purification of Chitinase from *E. coli* XL1-Blue pTrc99A/pKCHI23-3

The enzyme was purified to homogeneity with a final yield of 6.4%. A summary of the purification is given in Table 1. The specific activity of the crude enzyme from *E. coli* XL1-Blue pTrc99A/pKCHI23-3 was 14-fold higher than that of the crude enzyme from *T. vulgaris*. The specific activity of the enzyme purified from *E. coli* XL1-Blue pTrc99A/pKCHI23-3 (318 units/mg) was lower than that of the enzyme purified from *T. vulgaris* KFB-C100 (Yoon *et al.*, unpublished data). The enzyme from *E. coli* XL1-Blue pTrc99A/pKCHI23-3 was probably partially inactivated during heat treatment at 70°C in the purification procedure.

Molecular Determination of the Purified Enzyme

The molecular mass of the recombinant enzyme was estimated at about 59 kDa by gel filtration on a TSK-Gel HW-55F column (Fig. 7A). The analysis by SDS-PAGE indicated the molecular weight of the polypeptide to be 59 kDa (Fig. 7B), suggesting that the recombinant enzyme is composed of a monomer. The molecular weight of the recombinant enzyme was identical with that of the native enzyme (data not shown).

Substrate Specificity of Chitinase from Recombinant Cells

The substrate specificity of the recombinant enzyme was studied with chitin and various chitin-relative substrates (Table 2). Powdered chitin, colloidal chitin, and glycol chitin served as good substrates. However, various other polysaccharide compounds, such as cellulose and laminarin, were not hydrolyzed by the enzyme. The enzyme did not hydrolyze *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (*p*NPGlcNAc), a substrate of exo-chitinase, which indicated that the enzyme was an endo-chitinase. A thermostable chitinase from *Bacillus licheniformis* KFB-C14 could not hydrolyze chitosan at all [7], whereas, the new thermostable enzyme described here had the ability to catalyze chitosan. The recombinant and native enzymes showed similar substrate specificity. The K_m values of the recombinant enzyme were similar to those of *T. vulgaris* KFB-C100 (7.0 mg/ml for chitin and 8.8 mg/ml for colloidal chitin) and higher than that of *T. vulgaris* for glycol chitin (5.4 mg/ml).

Hydrolysis Products of Colloidal Chitin

We observed the change of hydrolysis products of colloidal chitin during incubation with the recombinant enzyme at 55°C for 12 h. In the hydrolysis products of colloidal chitin, $(GlcNAc)_2$ was detected as a major product (Fig. 8). The reaction product of the cloned enzyme was the same as that of the native enzyme. Substrate specificity and catalytic efficiency of the cloned enzyme

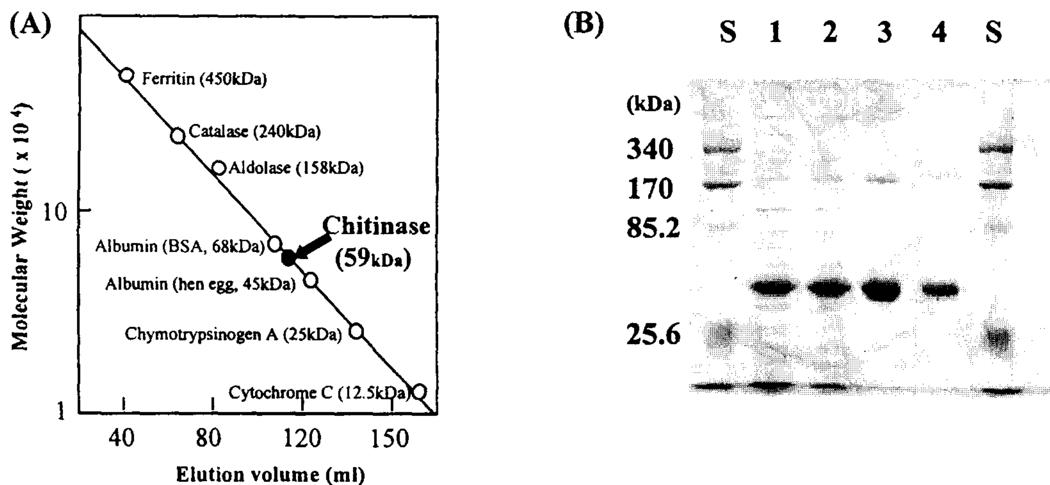

Fig. 7. Determination of the molecular weight of chitinase from *E. coli* XL1-Blue pTrc99A/pKCHI23-3 by gel filtration (A) and SDS-PAGE (B).

Lane S, size marker; lane 1, after ammonium sulfate fractionation; lane 2, after heat treatment at 70°C for 30 min; lane 3, after Butyl-Toyopearl 650 M Chromatography; lane 4, after TSK-Gel HW-55F filtration.

Table 2. Substrate specificity of chitinase from *E. coli* XL1-Blue pTrc99A/pKCHI23-3.

Substrate (1.0%)	Relative activity (%)	V_{max} (μmol/h/mg)	K_m (mg/ml)
Chitin[a]	100	335	7.9
Colloidal chitin	79	268	8.3
Glycol chitin	62	218	7.0
Soluble chitosan	8.0	-	-
Glycol chitosan	5.0	-	-
Chitobiose	0	-	-
Chitotriose	0	-	-
*p*NPGlcNAc[b]	0	-	-
Cellulose	0	-	-
Laminarin	0	-	-

[a]Purified powder (from Sigma), [b]*p*-Nitrophenyl-β-D-*N*-acetylglucosaminide.
-: not applicable.

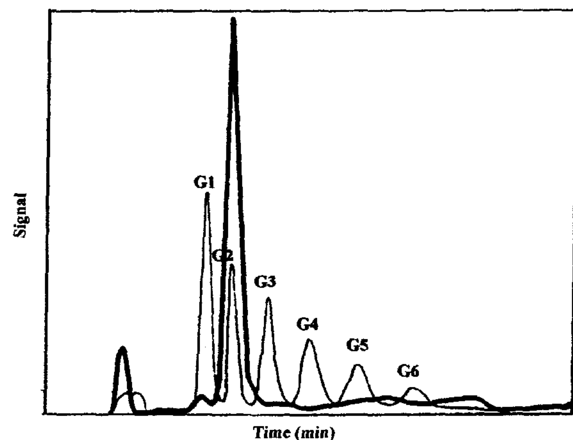

Fig. 8. HPLC of the products produced in hydrolysis of colloidal chitin by chitinase from *E. coli* XL1-Blue pTrc99A/pKCHI23-3.

Hydrolysis was performed for 12 h at 55°C. G1-6 indicates standard $(GlcNAc)_n$ (n = 1~6).

were the same as those of the native enzyme as well. Endo-type chitinases have been purified from several microorganisms. Although the properties of the enzyme of *T. vulgaris* are generally similar to those of other chitinases, the chitinase of this microorganism differs from other chitinases in thermostability and the hydrolysis pattern of colloidal chitin. The chitinase activity of *T. vulgaris* remained 100% after heating at 50°C for 30 min. About 75% of the initial activity remained after heating at 80°C for 10 min and 50% after 30 min. Thus, chitinase of *T. vulgaris* is a very thermostable enzyme. The enzymatic degradation product of colloidal chitin was mainly chitobiose, suggesting that further separation processes would not be necessary. On the other hand, the endo-chitinases isolated from various microorganisms produced chitooligomers of several sizes. Chitinase I from *Bacillus licheniformis* X-7u hydrolyzed colloidal chitin to mainly chitobiose, however, other properties (for example, molecular weight and thermostability, etc.) were different from those of the enzyme of *T. vulgaris* [18]. It has been reported that *B. licheniformis* KFB-C14 produced a thermostable chitinase [7]. However, substrate specificity of the cloned enzyme was different from that of the enzyme from *B. licheniformis* which could not hydrolyze chitosan. The hydrolysis products of colloidal chitin by the cloned enzyme were also different from those by the other endo-type enzymes. Therefore, further studies on structure-function relationships should be helpful in elucidating the differences. We are now determining the DNA nucleotide sequence of the gene.

Acknowledgments

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