

Cloning of alkaline protease gene from *Bacillus subtilis* 168

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Abstract

The aim of this study was to clone the serine alkaline protease-encoding gene from *Bacillus subtilis* 168. This protease, which can have many applications especially in detergent, may be industrially an important enzyme. For the amplification of the gene, PCR was performed with a pair of primers specifically designed for this purpose. Electrophoresis of the PCR product showed the expected band of 1329 bp. Restriction analysis also confirmed the integrity of the PCR product. After ligation of amplified gene into pTZ57R by the method of TA cloning, digestion with appropriate restriction enzymes confirmed the integrity of the cloned gene. Successful cloning of the protease gene from *B. Subtilis* could pave the way for the expression studies in a suitable host.

Keywords: Cloning; Alkaline protease; Industrial enzyme; *Bacillus subtilis* 168

INTRODUCTION

Proteases are one of the most important industrial enzymes that are used in many industries, such as detergent, food, pharmaceutical, textile and leather industry (1-3). Alkaline proteases that are active in alkaline pH and high temperatures are industrially more important, especially in detergent industry (3-5). Although these enzymes have been found in many viruses, fungi and animals, bacterial alkaline proteases have more importance (1,6). Among the bacteria, many species of the genus *Bacillus* are widely used in industrial production of proteases (7).

Bacillus subtilis 168 as a member of this genus that can produce several proteases is also used as a host organism for cloning. This is a gram-positive aerobic rod-shape bacterium with ability to produce endospore and lives in water, soil and plant residues (8,9). *B. subtilis* 168 produces a variety of extracellular proteases at the end of the exponential phase of its growth (10,11). This bacterium also produces an intracellular alkaline protease called alkaline protease X. This protease that is

expressed in stationary phase of bacterial growth has significant similarity to subtilisins, thermitases and pyrolysins (12). As mentioned above, proteases isolated from bacteria have widespread industrial uses. In this study the protease gene from *Bacillus* species was cloned for future production of this enzyme in our laboratory.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *B. subtilis* 168 used in this study was purchased from DSMZ (Germany, DSM No. 402). *E. coli* Hb101 as the host cell was provided by Cinnagen (Tehran, Iran). Oligonucleotide primers were synthesized by Fazabiotech (Tehran, Iran). Taq polymerase was purchased from BioRon (Tehran, Iran). The vector pTZ57R, DNA ligase, DNA molecular size marker, *Hind*III digested λDNA and all restriction enzymes were from Fermentas (Tehran, Iran). High Pure PCR template preparation kit, QIA quick gel extraction kit and Ins T/Aclone PCR product cloning kit were prepared from Roche, QiaGen

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and Fermentas, respectively. All other chemicals were from Merck (Tehran, Iran).

Amplification of the gene by PCR

After the overnight culture of *B. subtilis* 168 in LB broth, the genome of the bacterium was extracted by High Pure PCR Template Preparation Kit. PCR was performed using the forward primer (22mer, 5'-CATATGTTTGGGTACTCTATGG-3') and the reverse primer (25mer, 5'-GGATCCTTATTGGCCGGAACGGAA-3'). Two µl of *B. subtilis* extracted genome (60 ng) was amplified in a 50 µl reaction mixture containing 2.5 µM of each primers, 5 u of Taq polymerase, 0.5 mM each deoxyribonucleotide triphosphate and 10X-PCR buffer (containing 2 mM MgCl₂). The thermocycling profile was as follow: initial denaturation at 94 °C for 5 min; 35 repeated cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min; and a final extension of 72 °C for 20 min. The PCR product was analyzed using 0.8% agarose gel electrophoresis and digestion with restriction enzymes at 37 °C for 1 h.

Construction of the cloning vector containing alkaline protease gene

The PCR product was extracted from the 0.8% agarose gel using QIA quick Gel Extraction Kit. The amplified gene was inserted into pTZ57R cloning vector by TAcloning method. For this purpose the insert and vector (molar ratio of 3/1, respectively), DNA ligase (1 u) and its buffer were added at final volume of 10 µl and incubated at 16 °C overnight. Transformation was carried out by heat shocking the *E. coli* Hb101 competent cells prepared by CaCl₂ method 39 °C for 1 min (13). After the screening of transformed colonies on LB agar-ampicillin culture medium, plasmid preparation was accomplished by alkaline lysis method (13). The recombinant plasmids were confirmed with appropriate restriction enzymes.

RESULTS

Amplification of the gene

After the electrophoresis of the PCR product, a band of approximately 1300 bp was observed as shown in Fig. 1. The PCR product was then digested by *Xba*I and *Eco*RI enzymes and was run on 0.8% agarose gel. Restriction enzymes analysis showed two bands of approximately 500 bp and 800 bp for *Xba*I, and two bands of nearly 250 bp and 1100 bp for *Eco*RI as shown in Fig. 2.

Construction of the cloning vector

After ligation and transformation, the product was spread on LB agar-ampicillin and incubated in 37 °C for an overnight to screen the transformed colonies. Fifty seven colonies were observed on plate. Plasmid preparation was performed for a number of colonies. To screen the recombinant plasmids, the prepared plasmids were digested by *Eco*RI. Two bands about 1100 bp and 3000 bp were observed as shown in Fig. 3. Further analysis was carried out by double digestion with *Nde*I and *Bam*HI, causing isolation of insert from vector. The electrophoresis showed two bands of approximately 1300 bp and 3000 bp as in Fig. 4.

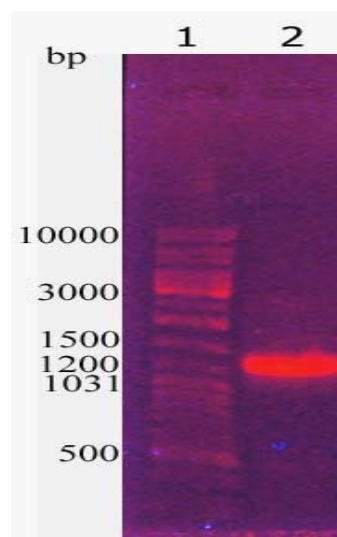


Fig. 1. Electrophoresis of the PCR product of alkaline protease gene from *Bacillus subtilis* 168. After PCR, 5 µl of the product was run on 0.8% agarose gel electrophoresis. The expected band of approximately 1300 bp was observed. Lane 1: DNA size marker 100 bp, Lane 2: PCR product sample.

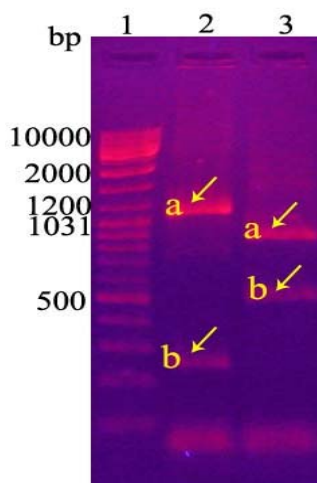


Fig. 2. Analysis of the PCR product of alkaline protease gene with restriction enzymes. For confirmation of the correctness of the amplified gene two sample of it were digested with *EcoRI* and *XbaI* enzymes, separately and then were run on 0.8% agarose gel electrophoresis with a DNA size marker 100 bp. By the enzyme *EcoRI*, two bands of approximately 1100 bp and 250 bp and by *XbaI* two bands of nearly 800 bp and 500 bp must be shown. Lane 1: DNA size marker 100 bp, Lane 2: Digested sample of Apr gene with enzyme *EcoRI*, Lane 3: Digested sample of Apr gene with enzyme *XbaI*.

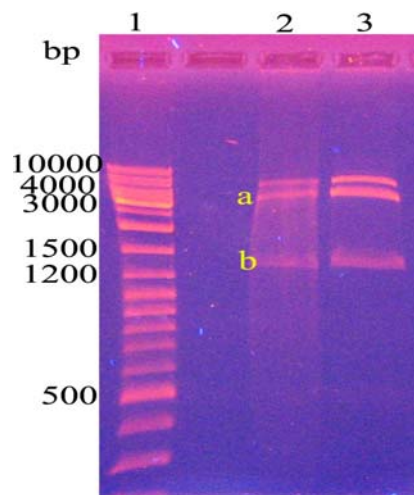


Fig. 4. Analysis of pTZ57R/alkalinepro plasmids prepared from colonies with *NdeI*-*BamHI* double digestion. Further screening of the recombinant pTZ57R/ alkalinepro plasmids was performed by double digestion with *NdeI* and *BamHI* enzymes and then the samples were run on 0.8% agarose gel electrophoresis with a DNA size marker 100 bp. Two bands about 3000 bp and 1300 bp must be observed after digestion, representing the vector and the isolated insert respectively. Lane 1: DNA size marker 100 bp, Lane 2 and 3: Digested pTZ57R/ alkalinepro plasmids with both *NdeI* and *BamHI*.

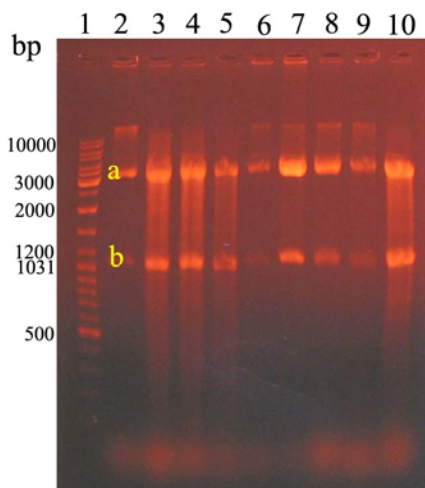


Fig. 3. Analysis of pTZ57R/alkalinepro plasmids prepared from colonies with *EcoRI*. To screen the recombinant pTZ57R/alkalinepro plasmids, the plasmids prepared from colonies were digested by *EcoRI* and then were run on 0.8% agarose gel electrophoresis with a DNA size marker 100 bp. Two bands about 3000 bp and 1100 bp must be observed after digestion. Lane 1: DNA size marker 100 bp, Lane 2-10: Prepared and digested pTZ57R/alkalinepro plasmids with *EcoRI*.

DISCUSSION

The aim of this study was to clone the alkaline protease gene of *B. subtilis* 168. The size of PCR product conformed to expected size i.e. 1329 bp. The results obtained from digestion of the PCR product with the restriction enzymes confirmed the correctness of gene amplification. After cloning of the gene, restriction of the plasmid product with appropriate enzymes confirmed the integrity of cloning. Isolation of insert from vector using double digestion method further confirmed the correctness of cloning.

As we discussed before, many studies have been performed in cloning of alkaline protease gene from *Bacillus* species. The gene encoding subtilisin Carlsberg from *B. licheniformis* have been cloned in pBR322 vector by Jacob et al. (1985) that has a 1137 bp open reading frame (ORF) encoding 379 aa (14). Peng et al. (2003) have cloned the subtilisin DFE gene from *B. amyloliquefaciens* DC-4 in pGEM-T plasmid that has a 1146 bp ORF encoding 382

aa (15). Also *B. lentus* and *B. alkalophilus* alkaline protease genes have been cloned and sequenced which both encode 380 aa proteins (7,16). As the sequence analysis of alkaline proteases of subtilase super family shows, almost all of them have total length of about 380 aa.

In this study we could clone the *B. subtilis* 168 alkaline protease X gene in pTZ57R cloning vector by the fast method of TAcloning. This enzyme is a member of subtilisin like protease family with a 1326 bp ORF encoding a 442 aa protein.

REFERENCES

1. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Bio Rev.* 1998;62:597-635.
2. Fathi Najafi M, Deobagkar D, Deobagkar D. Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. *J Bacteriol.* 2005;8:197-203.
3. Beshay U. Production of alkaline protease by *Tredinobacter turnirae* cells immobilized in Calcium alginate beads. *Afr J Biotechnol.* 2003;2:60-65.
4. Maurer KH. Detergent proteases. *Curr Opin Biotechnol.* 2004;15:330-334.
5. Kumar CG, Takagi H. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnol Adv.* 1999;17:561-594.
6. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol.* 2002;59:15-32.
7. Jorgensen PL, Tangney M, Pedersen PE, Hastrup S, Diderichsen B, Jorgensen ST. Cloning and sequencing of an alkaline protease gene from *Bacillus lentus* and amplification of the gene on the *B. lentus* chromosome by an improved technique. *Appl Environ Microbiol.* 2000;66:825-827.
8. Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, et al. The complete genome sequence of gram-positive bacterium *Bacillus subtilis*. *Nature.* 1997;390:249-256.
9. Rivolta C, Pagni M. Genetic and physical maps of the *Bacillus subtilis* chromosome. *Genetics.* 1999;151:1239-1244.
10. Stephenson K, Bron S, Harwood CR. Cellular lysis in *Bacillus subtilis*; the affect of multiple extracellular protease deficiencies. *Appl Microbiol.* 1999;29:141-145.
11. Kodama T, Endo K, Ara K, Ozaki K, Kakeshita H, Yamane K. Effect of *Bacillus subtilis* *spoOA* mutation on cell wall lytic enzymes and extracellular proteases, and prevention of cell lysis. *Bioscience.* 2007;103:13-21.
12. Valbuzzi A, Ferrari E, Albertini AM. A novel member of the subtilisin-like protease family from *Bacillus subtilis*. *Microbiology.* 1999;145:3121-3127.
13. Sambrook J, Russel DW. *Molecular cloning: A Laboratory Manual.* 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2002. p. 32-33, 116-118.
14. Jacobs M, Eliasson M, Uhlen M, Flock JI. Cloning, sequencing and expression of subtilisin Carlsberg from *Bacillus licheniformis*. *Nucleic Acids Res.* 1985;13:8913-8926.
15. Peng Y, Yang XJ, Xiao L, Zhang YZ. Cloning and expression of a fibrinolytic enzyme (subtilisin DFE) gene from *Bacillus amyloliquefaciens* DC-4 in *Bacillus subtilis*. *Res Microbiol.* 2004;155:167-173.
16. Van der Laan JC, Gerritse G, Mulleners LJ, Van der Hoek RA, Quax WJ. Cloning, characterization and multiple chromosomal integration of a *Bacillus* alkaline protease gene. *Appl Environ Microbiol.* 1991;57:901-909.