International Journal of Biological Macromolecules 50 (2012) 664–671

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Cloning and expression of alkaline protease genes from two salt-tolerant alkaliphilic actinomycetes in *E. coli*

S.D. Gohel, S.P. Singh*

Department of Biosciences, Saurashtra University, Rajkot 360 005, India

ARTICLE INFO

Article history:
Received 11 December 2011
Received in revised form 21 January 2012
Accepted 25 January 2012
Available online 3 February 2012

Keywords:
Salt tolerant alkaliphilic actinomycetes
Alkaline protease
Cloning and expression
Recombinant enzymes

ABSTRACT

Cloning and expression of recombinant alkaline serine proteases from two salt tolerant alkaliphilic actinomycetes strains: OM-6 (EU710555.1) and OK-5 (HM560975) were successfully obtained in mesophilic host, *Escherichia coli*. The positive clones harboring protease genes were selected on the basis of restriction analysis on agarose gel. The effect of temperature and IPTG concentrations on the expression of recombinant proteases and solubilization of the expressed enzymes was assessed. SDS-PAGE revealed protease bands corresponding to 25 kDa and 20 kDa molecular mass representing OM-6 and OK-5 proteases, respectively. Cloning and expression of alkaline proteases from salt tolerant alkaliphilic actinomycetes would pave the way for further biochemical and molecular characterization to achieve unexplored features of the biocatalysts from extremophiles.

1. Introduction

Proteases from extremophiles are the most important enzymes from commercial point of view as well as for understanding fundamental cellular features. Proteases are one of the largest selling enzymes accounting about 60% of the total enzyme market worldwide [1–3]. Alkaline proteases are generated by a wide range of organisms, including bacteria, actinomycetes, molds, yeasts, and mammalian tissues [4–8]. However, the ability to withstand the rigorous environments is not sufficient for commercial success. In addition, number of other factors must also be considered and investigated. Therefore, Recombinant DNA Technology in conjunction with the protein engineering tools is being extensively used to improve and evolve enzymes suitable for various unconventional applications.

Page and coworkers [9] chose *Streptomyces griseus* trypsin (SGT) as a model scaffold for the development of serine proteases with enhanced substrate specificity. Recombinant SGT has been produced in a *Bacillus subtilis* expression system in a soluble active form. A gene encoding an alkaline proteinase (subtilisin) was cloned and sequenced from alkaliphilic *Bacillus lentus* NCIB 10309 into *Bacillus subtilis* DN497 [10]. Similarly, the genes encoding chitinases were cloned and sequenced from alkaliphilic actinomycete, *Nocardiopsis prasina* OPC-131 into *Escherichia coli* [11]. Fernandez-Abalos et al. [12] demonstrated the post translational processing of the xylanase Xys1L from *Streptomyces halstedii* JM8 by secreted alkaline serine protease. Recently, a novel calcium independent serine protease from an alkaliphilic bacterium, *Nesterenkonia* sp. AL20, has been purified and crystallized by X-ray analysis [13].

Due to the advancement in molecular tools and increasing realization on the potential of the extremophiles, much of the work in this area is being done at molecular level to expand the horizon of genomics and proteomics in these organisms. Regulation of gene expression of various enzymes from extremophiles would pave the way for further molecular evolution to achieve unexplored and non-existed features of biocatalysts. Genes from the extremophiles often cloned and over expressed in domestic host systems to obtain large quantities of enzymes [14–16]. More recently alkaline protease genes from haloalkaliphilic bacteria and metagenomically derived from saline habitat of Coastal Gujarat were cloned, sequenced, and over expressed in functional state into *E. coli* [17,18]. The native and recombinant proteases were analyzed for structure and function relationship. However, since only few genes from salt tolerant alkaliphilic actinomycetes have been cloned and analyzed for their expression in heterologous hosts, cloning and expression of alkaline protease from these organisms would be a valuable addition to the knowledge of recombinant biocatalysis from extremophiles. Further, it would add to the molecular insights into the physicochemical properties of alkaline proteases for its functioning under multitude of extremities.

* Corresponding author at: Department of Biosciences, Saurashtra University, Rajkot 360 005, Gujarat, India. Tel.: +91 281 2586419; fax: +91 281 2586419.
E-mail address: satyapsingh@yahoo.com (S.P. Singh).

0141-8130/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijbiomac.2012.01.039

2. Materials and methods

2.1. Microorganisms

Alkaline proteases were purified from two halo-tolerant and alkaliphilic actinomycete strains OM-6 and OK-5 isolated from Okha-Madhi and Okha sites, respectively, from Coastal Gujarat, India by enrichment culture and standard serial dilution and plating methods. The medium used for the screening of protease producing microorganisms and the cultivation of strains consisted of (g/L: gelatin, 5; peptone, 5; yeast extract, 5; NaCl, 50). The pH of the medium was adjusted to 9 with 20% (w/v) Na_2CO_3 and cultivation was performed aerobically at 37 °C. For 16S rRNA gene sequencing, the genomic DNA of both the strains was subjected to consensus universal primers designed for 16S rRNA amplification. The PCR product was sequenced by using pair of forward, reverse, and internal primers. Sequence data were aligned and analyzed for closest homologous actinomycetes using Mega 3.1 using Neighbor Joining method. Further, cultural and biochemical characterization of isolates were carried out to confirm the results obtained by 16S rRNA sequencing.

2.2. Protease assay

Alkaline protease activity was measured by modified Anson–Hagihara's method [19]. The enzyme (50 μl) was added to 3.0 ml hammarsten casein (0.6%, w/v, in 20 mM NaOH-Borax buffer, pH 10) and the reaction mixture was incubated at 60 °C for 10 min. The reaction was terminated by the addition of 3.2 ml of TCA mixture (0.11 M trichloro acetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid) and incubated at 60 °C for 10 min. The precipitates were removed by filtration through Whatman-1 filter paper and absorbance of the filtrate was measured at 280 nm. One unit was defined as the amount of enzyme liberating 1 μg of tyrosine per minute at 60 °C under the standard assay conditions.

2.3. Genomic DNA extraction

For the DNA isolation, OM-6 was grown on YEME medium (Yeast Extract-Malt Extract broth) with 5% salt at pH 9.0 for 48 h at 30 °C. The extraction of genomic DNA was carried out in accordance with the methods described by Sambrook et al. [20]. The culture aliquots were centrifuged for 15 min at 4193 × *g* and cell pallets were suspended in STE buffer. The samples were again centrifuged at 4193 × *g* and the pellets were re-suspended in GET buffer with SDS (20%) and freshly prepared lysozyme solution (10 mg/ml in Tris–Cl, pH 8) followed by incubation for 2 h at 60 °C. After P: C: I (phenol: chloroform: isoamylalcohol) and C: I extraction steps, the DNA was pooled by adding 3 M potassium chloride and chilled ethanol. The DNA was then suspended in appropriate volume of miliQ water and preserved at −20 °C. The quality of the DNA was examined by spectrophotometry and agarose gel electrophoresis.

2.4. Primer designing and PCR amplification

According to the previously known whole sequence of the halophilic alkaline protease gene, six pair of primers was designed for cloning the alkaline protease gene in present study. Among the six primer pairs, four primers (SPS-1F/R, SPS-3F/R, SPS-4F/R, SPS-5F/R) were designed using known sequences of alkaline protease genes from *Bacillus halodurans*, *Bacillus cerus*, *Oceanobacillus iheyensis*, and haloalkaliphilic *Bacillus* sp. Two primer pairs (SPS-6F/R, SPS-7F/R) were designed on the basis of conserved sequences of Haliphilic *Bacillus* sp., using multiple sequencing tools followed by block generation with degenerate primer designing bioinformatics tool CODEHOP. Nucleotide sequence of each primer pair is shown in Table 1. For amplification of alkaline protease gene, the gradient PCR method was developed to specifically amplify protease sequences from OM-6 and OK-5 using Eppendorf Master Cycler Gradient with all six primer pairs. PCR mixture consisted of 100 ng of DNA as the template and 25 pmol of each forward and oligonucleotides primer (Sigma Aldrich, Life Sciences), 25 μl of 2× Red mix plus (Merk, Life Sciences). The PCR program consisted: 1 cycle of initial denaturation at 95 °C followed by 30 cycles at 94 °C for 30 s, gradient of annealing at 60 °C with gradient of 8 °C for 45 s, 72 °C for 1.5 min, and final extension step of 72 °C for 5 min. Standardization of annealing temperature was carried out by keeping gradients of temperature calculated on the basis of melting temperature (T_m) of the primer pair. PCR reaction was carried out at three annealing temperatures: 56.1, 59.8, and 63.7 °C using Gradient Thermocycler (Eppendorf). The amplified products were visualized on 0.8% agarose gel and stored at −20 °C till further use.

Table 1
Nucleotide sequence of each primer pair.

Primer designation	Sequence
SPS-1F	5′-gga tcc ttg aaa aac aaa atc att-3′
SPS-1R	5′-gtc gac tta aga agc ttt att taa c-3′
SPS-3F	5′-gga tcc ttg aaa aca aaa tca ttg-3′
SPS-3R	5′-gtc gac tta aga agc ttt att taa c-3′
SPS-4F	5′-gga tcc cta ctt gat gta ga-3′
SPS-4R	5′-gtc gac atg cat atc gga aaa c-3′
SPS-5F	5′-gga tcc gcc gcc gag gac gac-3′
SPS-5R	5′-gtc gac atg gga tat tat gac-3′
SPS-6F	5′-gga tcc gcc gcc gag gac gac-3′
SPS-6R	5′-gtc gac gga cca gac cgt cg-3′
SPS-7F	5′-cat atg ccg ccg agg agg ac-3′
SPS-7R	5′-gtc gac ggc ctt cgt gtg g-3′

2.5. Construction of recombinant clones

2.5.1. Plasmid isolation and restriction digestion

Plasmid DNA was isolated by SDS mini preparation method. *E. coli* Top_{10}, harboring pET 21a$^+$ plasmid was inoculated in Luria Bertani (LB) broth containing 50 μg/ml of ampicillin at 37 °C. 3 ml of overnight grown bacterial culture was used for the extraction of plasmid. For restriction digestion, 50 ng pET 21a$^+$ vector/1 μg insert DNA, 2.5 μl 10× restriction enzyme buffer, 1–2 U each restriction enzyme, and *x* μl nuclease-free water to volume were assembled in a microcentrifuge tube to make total volume 25 μl and incubated at 37 °C for overnight. Followed by RE digestion, 5 μl digested product together with DNA markers was analyzed on agarose gel to evaluate the extent of digestion. Ethanol purification of amplicon and restriction endonuclease (RE) digested product was done after each reaction. Selection of right RE pair was decided on the basis of the bioinformatics based prediction for cutters and non-cutters sites within the vector and insert. Taking all parameters into consideration, *Bam*H I and *Sal* I sites were designed into primer pair combination to ensure cohesive end generation. pET 21a$^+$ was digested by *Bam*H I and *Sal* I as their presence was available in multiple cloning site (MCS), as well as they are among the list of non-cutters in alkaline proteases sequences. The inserts were gel-purified using cleangene PCR purification kit (Banglo Genei) to remove residual restriction enzyme and prevent self-ligation.

2.5.2. Preparation of competent cells and transformation

The competent cells preparation and transformation were carried out by standard calcium transformation procedures described by Sambrook et al. [20].