ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology, 3 (1): 49-55, 2004
ISSN 1682-2978

A Single Ser_{85}Ala Mutation Enhances the Catalytic Efficiency of Subtilisin E from *Bacillus subtilis* 168

Eliel R. Romero-García, Joel A. Esquivel-Naranjo, Norma Ramírez-Ramírez,
Jesús García-Soto and Mario Pedraza-Reyes
Institute of Investigation in Experimental Biology, Faculty of Chemistry,
University of Guanajuato, Noria Alta, S/N. Guanajuato, 36050, México

Abstract: A single mutation of Ser for Ala was introduced into the highly conserved 83-Gly-Val-Ala-85 region of AprE (subtilisin E) which resulted in the generation of a subtilisin mutant with an enhanced catalytic efficiency. The position of the mutation was placed in the conserved N-terminal end of the loop that connects a β-sheet with the α-helix containing the catalytic residue His_{64}. The mutant Ser_{85}Ala *apr*E gene was over expressed in a protease deficient *B. subtilis* genetic background and its product purified to homogeneity. The Ser_{85}Ala AprE protein exhibited a catalytic efficiency two fold higher than that of its wild type parent AprE due to a larger k_{cat}. Other biochemical properties such as thermal stability, optimum pH and temperature remained unchanged with respect to those exhibited by the pure wild type subtilisin. These results support the idea that mutations on the conserved stretch bend 83-Gly-Val-Ser-85 which connects two elements of secondary structure in AprE cause alterations on the catalytic properties of AprE and other subtilisins.

Key words: *Bacillus subtilis*, proteases, subtilisin E, site directed mutagenesis, enhancement of catalytic activity

INTRODUCTION

For many years microbial alkaline proteases have been extensively studied not only with the basic purpose of unraveling their catalytic mechanisms and structure function relationships but also for their potential industrial application as additives in detergents and food processing. Subtilisins produced by several species of the genus *Bacillus* have been widely characterized[1-3]. The *apr*E gene which encodes the major alkaline serine protease from *B. subtilis* 168 was cloned and sequenced[4]. Its DNA sequence showed to be 80% homologous to the *B. amylolyquefaciens* subtilisin gene and its translated coding sequence was shown to be 85% homologous to the protein sequence of subtilisin BPN'[4].

Practical applications of naturally occurring enzymes such as subtilisins have been limited, due in large part to relatively poor stability and catalytic activity under conditions that characterize industrial processes such as high temperatures, extreme pH, or non aqueous solvents. However, in addition to create new enzymes for applications in biotechnology, directed evolution methods can be used to explore the limits of protein function[5,6]. Moreover, directed random mutagenesis approaches have been used to alter various enzyme features, including thermal stability[7-10] alkaline stability[11] substrate specificity[12] and activity in dimethylformamide[5,13,14].

A protocol of in vivo random mutagenesis of *apr*E in a hypermutagenic *E. coli* genetic background and selection for hyperactive extracellular proteases in *B. subtilis* yielded a protease whose total activity in supernatant cultures was higher than that measured in the wild-type subtilisin E (Our unpublished results). DNA sequencing of the variant *apr*E gene revealed a double mutation event which resulted on a single Ser_{85}Ala substitution. In the present study, the residue Ser_{85} of AprE was changed to Ala by site directed mutagenesis. The mutant aprE gene was over expressed in *B. subtilis* from the IPTG inducible Spac promoter and the mutant Ser_{85}Ala protein was purified to homogeneity. Determination of the biochemical parameters of the mutant protein revealed that the amino acid replacement Ser_{85}Ala increases the catalytic efficiency of AprE due to an augmented k_{cat} value.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. *B. subtilis* and *E. coli* strains used in this study are listed in Table 1. Plasmids and cloned fragments are described in Table 2. Media used was Luria-Bertani[15]. When

Corresponding Auctor: Dr. Mario Pedraza-Reyes, IIBE. Bldg. "L". Faculty of Chemistry, University of Guanajuato, Noria Alta S/N. Guanajuato 36050, Gto. México, Tel: +52 473 73 2 00 06 (ext 8161), Fax: +52 473 73 2 00 06 (ext 8153) E-mail: pedrama@quijote.ugto.mx

Table 1: Bacterial strains used in this study

Bacterial strain	Genotype and description	Reference or source
Escherichia coli		
SURE	*e14*-(McrA-) Δ(*mcr*CB-*hsd*SMR-*mrr*)171 *end*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *lac rec*B *recJ sbc*C *umu*C::Tn*5* (Kan^r) *uvr*C [F'*pro*AB *lacI*^{q}*lacZ*ΔM15Tn*10* (Tet^r)]	Greener (1990)
XL1-BLUE	*rec*A1 *end*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *lac* [F'*pro*AB *lacI*^{q}*lacZ*ΔM15Tn*10* (Tet^r)]	Laboratory stock
Bacillus subtilis		
168	*trp*C2	W.L. Nicholson
1A751	*egl*SΔ*102 bgl*T/*bgl*SΔEV *apr*E *npr*E *his*	BGSC*
PERM170	*B. subtilis* 1A751 containing pPERM168 integrated into *amy*E locus	This study
PERM176	*B. subtilis* 1A751 containing pPERM175	This study
PERM551	*B. subtilis* 1A751 containing pPERM550	This study

*BGSC: *Bacillus* Genetic Stock Center

Table 2: Plasmids used in this study

Plasmid	Description	Reference or source
pDG148	*spac* expression vector, Amp^r, Kan^r	W.L. Nicholson
pUC19	High-copy vectors, Amp^r	Laboratory stock
pPERM161	1.7 kpb *Sma*I-*Bam*HI PCR product containing wild-type *apr*E cloned into pUC19	This study
pPERM167	pDG364 without *Nde*I restriction site	This study
pPERMI168	1.7 kpb *Sma*I-*Bam*HI PCR product containing wild-type *apr*E cloned into pPERM167	This study
pPERM172	1.2 kpb *Acc*I-*Xba*I fragment containing wild-type *apr*E without operator/promoter from pPERM161 subcloned into pDG148	This study
pPERM175	pPERM172 without *lac*I repressor	This study
pPERM241	0.75 *BamHI*/*Sph*I fragment of WT *apr*E from pPERM172 cloned in pAlter-1	This study
pPERM257	Same as pPERM241 but with Ser_{85}Ala mutation in *aprE*	This study
pPERM550	0.75 kpb *Hind*III-*Sph*I fragment from pPERM257 subcloned into pPERM172.	This study

appropriate, antibiotics were added to media at the following final concentrations: chloramphenicol (Cm), 5 μg ml^{-1}; ampicillin (Amp), 100 μg ml^{-1}; tetracycline (Tet), 15 μg ml^{-1} and kanamycin (Kan), 20 μg ml^{-1}. Cells were grown in liquid media with vigorous aeration or on solid media at 37°C.

Genetic and molecular biology techniques: Preparation of competent *E. coli* or *B. subtilis* cells and their transformation with DNA was performed as described elsewhere[16,17]. Extraction of chromosomal DNA from *B.subtilis* was carried out according to the protocol of Cutting and Vander Horn[18]. Small scale preparation of plasmid DNA from *E. coli* cells, enzymatic manipulations and agarose gel electrophoresis were performed by standard techniques[16]. Large scale preparation and purification of plasmid DNA was accomplished using commercial ion exchange columns, according to the instructions of the supplier (QIAGEN Inc, Valencia, CA). DNA plasmids purified through QIAGEN columns were processed for sequencing in a Perkin Elmer (Norwalk, CON) model 377A automated DNA sequencer.

DNA amplification, cloning and sequencing of *apr*E from *B. subtilis* 168: The complete *apr*E gene was amplified by PCR, using genomic DNA from *B. subtilis* I168 as template and the oligonucleotide primers 5′-GGCCCGGGGGCGGCCGCATCTGATG-3′ (forward) and 5′-GGGGATCCGCGCCGGAACATCAGG-3′ (reverse). The primers were designed to insert *Sma*I and *Bam*HI sites (underlined) to facilitate cloning into the pUC19 and pDG364 vectors. Amplification was performed on 0.1 μg of chromosomal DNA using a MJ. Research (Watertown, MA) Minicycler with Vent DNA polymerase (New England Biolabs, Beverly, MA), according to the manufacturer′s recommendations. The 1.7 kpb PCR fragment was digested with *Sma*I and *Bam*HI and ligated into both pUC19 and pPERM167 to generate pPERM161 and pPERM168, respectively which were replicated in *E. coli* SURE. The amplified *apr*E gene present on pPERM161 was sequenced on both strands.

Site directed mutagenesis of AprE: A 0.75 kpb *Hind*III-*Sph*I fragment of encoding part of the wild type mature subtilisin E was removed from pPERM172 (Table 2) and inserted into *Hind*III-*Sph*I-cleaved plasmid pALTER-1 (Promega, Madison, Wis) to create plasmid pPERM241. Directed mutagenesis was performed with the altered sites II *in vitro* mutagenesis system (Promega, Madison, Wis) according to the instructions of the manufacturer. The mutagenic primer used was: 5'-GC GCT TGG CGC AAC GCC CAG-3′ o replace Ser_{85} for Ala. The construction

carrying the 0.75 kbp *Hind*III-*Sph*I mutant fragment from *apr*E was identified by nucleotide sequencing and named pPERM257. The 0.75 kbp *Hind*III-*Sph*I fragment of pPERM175 (pDG148 carrying the ORF of WT *apr*E, Table 2) was replaced with the 0.75 kbp *Hind*III-*Sph*I from pPERM257, carrying the mutations $Ser_{85}Ala$, to create pPERM550. The construction was introduced by transformation into the protease deficient strain *B. subtilis* 1A751 generating the *B. subtilis* strain PERM551.

Expression of *apr*E and *aprE* Ser_{85}Ala in *B. subtilis* IA751: A plasmid to express *apr*E from the *spac* promoter of pDG148 was constructed as follows. A blunt ended-1.2 kbp *Acc*I-*Xba*I fragment of *apr*E from pPERM161 was cloned into the *Hind*III site of pDG148 (Kindly provided by Wayne Nicholson, University of Arizona) previously treated with Klenow to generate plasmid pPERM172. This plasmid was digested with *Bam*HI to eliminate the *lac*I coding sequence resulting in plasmid pPERM175. Expression of the *aprE* $Ser_{85}Ala$ gene was performed in the strain pERM551, constructed as described above.

Purification of AprE and AprE Ser_{85}Ala: Purification of both AprE and AprE $Ser_{85}Ala$ was achieved utilizing the same following protocol. Unless otherwise stated, all purification procedures were performed at 4°C. Both, *B. subtilis* PERM 176 and 551 (Table 1) were grown in LB medium supplemented with Kan for 24 h. Expression of *aprE* $Ser_{85}Ala$ was induced by adding IPTG (5 mM) to the culture of *B. subtilis* PERM 551, during logarithmic growth. Culture broths were centrifuged (7000 rpm, 45 min) and three volumes of cold ethanol were added to the supernatant. After centrifugation (7000 rpm, 45 min), the precipitate was dissolved in 24 ml of 10 mM sodium phosphate buffer, pH 6.2 and dialyzed overnight against 2 liters of the same buffer. The dialyzed solution was applied to a 50 ml CM-BioGel-A column previously equilibrated with 10 mM sodium phosphate buffer, pH 6.2. The column was washed with 5 volumes of 10 mM sodium phosphate buffer, pH 6.2 and the enzyme was eluted with 250 ml of a linear 0.0-0.2 M NaCl gradient in the same buffer. The active fractions were lyophilized, resuspended in 0.5 ml of 10 mM sodium phosphate buffer, pH 6.2 and loaded onto a Superdex HR200 column (Pharmacia). The enzyme was eluted from the column with 10 mM sodium phosphate buffer, pH 6.2 and the peak fractions were analyzed on a 12% SDS-polyacrylamide gel[19] stained with Coomassie blue R-250

Enzyme and protein assays: The kinetic constants, k_{cat} and k_m of both AprE and AprE $Ser_{85}Ala$ were calculated from their double reciprocal plots. To this end, enzyme assays were performed utilizing different concentrations (from 0.3 to 5 mM) of the chromogenic substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPF) in a solution containing 100 mM Tris-HCl, pH 8.5 and 10 mM $CaCl_2$ at 37°C. The reactions were initiated by addition of pure enzyme (1 ng μl^{-1}). The amount of released *p*-nitroaniline was measured by absorbance at 450 nm. Protein was measured by the method of Lowry *et al.*[20].

RESULTS

The *apr*E locus including the structural gene as well as the operator/promoter region[4] was amplified by PCR from *B. subtilis* 168 and replicated into *E. coli* by subcloning into pUC19 to create pPERM161 (Table 2). The 1.7 kbp PCR fragment cloned into the insertion vector pDG364 (Table 2), was capable of directing the synthesis and excretion of AprE from the *amy*E locus of the protease deficient genetic background *B. subtilis* IA751 (Results not shown)

In a previous study, a protocol of directed evolution was applied to enhance the proteolytic activity of the wild type AprE enzyme from *B. subtilis* 168. This approach yielded a protease whose total activity in supernatant cultures was higher than that detected for the wild-type subtilisin E (Our unpublished results). Sequencing of the mutant *apr*E gene revealed the replacement of Ser by Ala at the amino acid position 85 (Fig. 1).

To better investigate the consequences of the mutation $Ser_{85}Ala$ in AprE, a site directed mutagenesis protocol was applied to exclusively insert this mutation into the *apr*E gene. The mutant AprE $Ser_{85}Ala$ enzyme was purified from the culture medium of a *B. subtilis* over-producer strain containing an IPTG inducible *spac-apr*E $Ser_{85}Ala$ construction into the plasmid pDG148. The excreted recombinant protease was purified by ion exchange using a CM-cellulose column followed by molecular sieving onto a Superdex HR 200 column as described in Materials and Methods. The same protocol was used to purify the wild-type enzyme from the culture medium of the over-producer strain *B. subtilis* PERM 176, harboring a constitutively induced *spac-apr*E construction. Following this protocol, we obtained pure samples of both the wild type AprE (Fig. 2, Lanes 1and 2) and the variant AprE $Ser_{85}Ala$ (Fig. 2, Lanes 3 and 4) enzymes as revealed by SDS-PAGE analysis of the fractions recovered from the last step of purification.

The kinetic constants k_{cat} and k_m for both the wild type AprE and AprE $Ser_{85}Ala$ enzymes were calculated from initial rate measurements of hydrolysis of AAPF. The change of the Ser_{85} residue by Ala in AprE did not alter

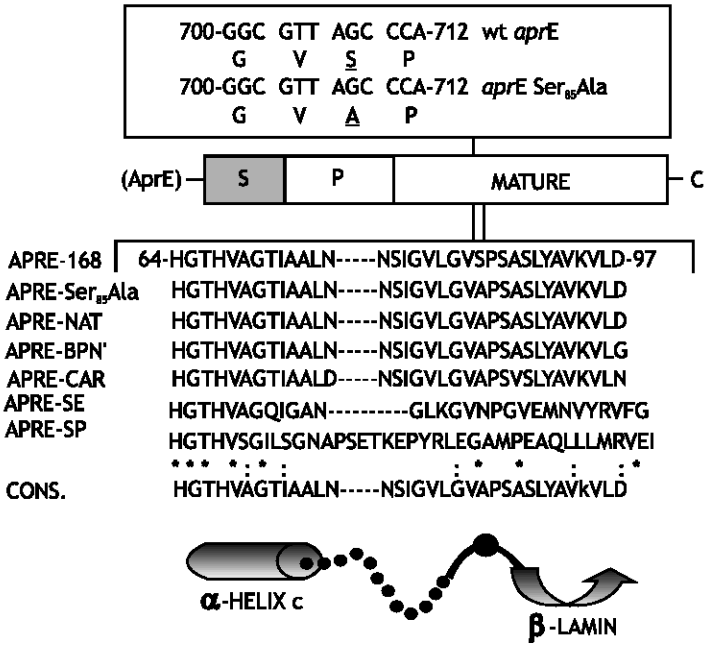

Fig. 1: comparison between the DNA sequences in the GVA region of *apr*E and *apr*E $Ser_{85}Ala$. Middle, Alignment of the AprE $Ser_{85}Ala$ region with the primary sequences of several subtilisines. AprE-168[4]; APR-NAT[21]; SUB-BPN'[27]; SUB-CAR[22]; EPIP-SE[23]; SCPA-SP[24]. Identical residues are indicated by *asterisks* and conserved changes by *colons*. Below, schematic representation of the secondary structure of AprE around the position of the mutation $Ser_{85}Ala$ of AprE $Ser_{85}Ala$. Dashed and solid lines indicate variable and conserved regions on the connecting loop, respectively

Table 3: Kinetic parameters of AprE and AprE $Ser_{85}Ala$ during hydrolysis of AAPF

Subtilisin E	K_{cat} (s^{-1})	K_m (mM)	K_{cat}/Km (s^{-1} mM^{-1})
WT AprE	23.5	1.5	15.6
AprE $Ser_{85}Ala$	46.0	1.5	30.6

the v_{max} of the hydrolytic reaction with respect to that of the wild type enzyme. However, the mutant enzyme exhibited a catalytic rate constant (k_{cat}) two times higher than that exhibited by AprE (Table 3). As a consequence of this change, the catalytic efficiency calculated for the AprE$Ser_{85}Ala$ mutant, exceeded that of the wild-type enzyme by a factor of 2. To further investigate other changes in the biochemical behavior of AprE $Ser_{85}Ala$, we evaluated the pH and temperature profiles, as well as thermal stability in both the variant and the wild type enzyme. Results revealed that the mutant retained the same biochemical properties observed for AprE. Thus, both enzymes showed a maximum activity against AAPF at a pH value of 9.0; maximum hydrolysis of AAPF by the pure enzymes occurred at 55°C (Results not shown). On the other hand, both the wild type and AprE 196 enzymes, maintained between 65-70% of maximum activity after 2 h of incubation at 50°C (Results not shown).

DISCUSSION

We inserted a $Ser_{85}Ala$ mutation on the *B. subtilis* 168 *apr*E gene by directed mutagenesis to investigate the catalytic consequences of this replacement on the AprE enzyme. Although an AprE mutant possessing the substitution $ser_{85}Ala$ by means of either site directed or random mutagenesis has not been previously reported, results of multiple sequence alignments revealed that 32 out of 35 subtilisins found in nature contain an Ala residue in the position 85[2]. Interestingly, naturally existing subtilisins such as AprE from *B. subtilis* 168[4], EpiP from *Staphilococcus epidermis*[23] and ScpA from *Streptococcus piogenes*[24] possess Ser, Asn or Met residues, respectively at that position.

Subtilisin E has been used as a target for protein design; for instance, Chen and Arnold[13,14] and You and Arnold[5] applying sequential rounds of *in vitro* random mutagenesis succeeded in enhancing the activity of AprE in the organic medium dimethylformamide. The evolved enzyme accumulated 13 amino acid substitutions on its surface, i.e., in the face that harbors the active site and substrate binding pocket[5]. Although most of the

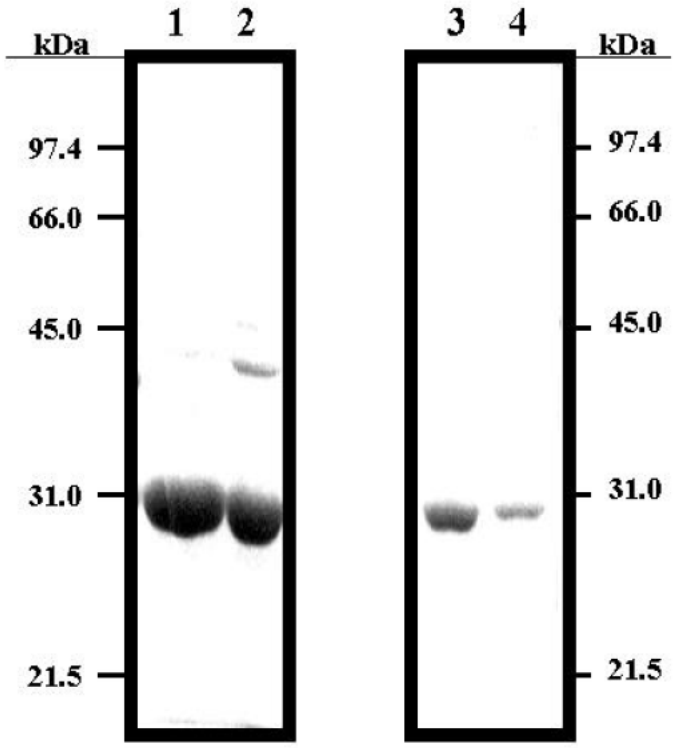

Fig. 2: SDS-PAGE analysis of protein samples taken from the peak of active fractions of AprE (Lanes 1 and 2) and AprE Ser_{85}Ala (Lanes 3 and 4) purified by FPLC through a Superdex HR200 column

substitutions were located in variable regions of connecting loop structures, three of them were found to be lying in conserved elements of secondary structure[5]. In contrast to these results, we found that the mutation Ser_{85} Ala in AprE196 was located on the other face of the enzyme in close contact with the residue His_{64}. The secondary structural topology of subtilisins[2] revealed that the position of the substitution Ser_{85}Ala lies within the conserved stretch bend GVA (residues 83-85). As shown in Fig. 1, this stretch of amino acids is located in the C-terminal edge of the loop that connects the β-lamin e3 with the α-helix c that contains the catalytic residue His_{64}[2]. Although most of the sequence of this connecting loop seems to be dispensable for activity[2] modification of the conserved GVA sequence results in dramatic alterations of the catalytic properties of subtilisins. This fact is supported not only by our results but also by those of Kano *et al.*[25] who observed that an AprN' variant adapted to function at low temperatures, was the result of a single substitution of the residue Val_{84} for Ile. A detailed analysis of the kinetic parameters on this mutant enzyme revealed an increase on its Km value[25].

Comparison of the kinetic parameters between purified AprE Ser_{85}Ala and wild type AprE, revealed a two fold increase in the catalytic efficiency of the former. As shown in Table 3, the augment in the catalytic efficiency of the mutant AprE Ser_{85}Ala obeyed to an increase in its k_{cat}, as its k_m value remained unchanged with respect to that exhibited by the wild type enzyme. Taking into account that Ser_{85} is not part of the active site of subtilisins[2] it was feasible to anticipate no alterations on the specificity of AprE as a consequence of the mutation Ser_{85}Ala. However, as discussed above a mutation on the neighbor residue Val_{84} to Ile on the sequence 83-GVA-85 of AprN' had a positive effect on its Km and as a result of the mutation the variant enzyme was adapted to operate at low temperatures. In contrast to these results, we found no alterations in other biochemical properties such as thermal stability, pH and temperature profiles between AprE196 and AprE.

Despite of differences on their codifying DNA primary structures, the amino acid sequence of AprE196[4] perfectly matched that of the subtilisin AprN produced by the strain *Bacillus subtilis* natto, NC2-1[21]. Although the kinetic parameters of AprN are currently unknown, it has been reported that both, subtilisin Carlsberg[22] and BPN'[27] which harbor the replacement Ser_{85}Ala possess higher activities than that exhibited by the wild type AprE[1]. Together with our results this evidence strongly supports the idea that the Ser_{85}Ala replacement contributes in augmenting the catalytic activity of subtilisins. As pointed out above, two other naturally

existing subtilisins, namely, EpiP[23] and ScpA[24] replace their residue 85 for Asn and Met, respectively however, the consequences of these replacements on the enzyme kinetics remain to be established.

Upon the lacking of differences between the specificities of AprESer$_{85}$Ala and AprE, during the hydrolysis of the synthetic substrate AAPF, the question arises as to why the former increased its k_{cat}. A careful analysis of the three dimensional structure of subtilisin E[26] reveals that the amino acid 85 in the sequence 83-GVS-85, besides being in close contact with His64, exists in a hydrophobic microenvironment. Therefore, the replacement of Ser$_{85}$, a polar aminoacid, for Ala into this microenvironment most possibly promotes disturbances which may affect the distances between the residues of the catalytic triad, such a structural change must speed up the proton transfer from Ser221 to His64 during enzyme catalysis.

Current experiments in our laboratory analyze the catalytic effects that suffers AprE following the elimination of both the conserved stretch bend 83-GVS-85 and the full loop that connects the β-lamin e3 with the α-helix c.

ACKNOWLEDGMENTS

This work was supported by grants 0462P-N and 31767-N from the Consejo Nacional de Ciencia y Tecnología (CONACYT) to M. Pedraza-Reyes. J.A. Esquivel-Naranjo and Eliel R. Romero-García were supported by Doctoral fellowships from CONACYT.

REFERENCES

1. Markland, F.S., Jr. and E.L. Smith, 1971. In: Boyer P.D. (Ed.), Subtilisins: primary structure, chemical and physical properties: The Enzymes. Academic Press Inc., NY., pp: 561-608.
2. Siezen, R.J., W.M. de Vos, J.A. Leunissen and B.W. Dijkstra, 1991. Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Engineering, 7: 19-37.
3. Siezen, R.J. and J.A. Leunissen, 1997. Subtilases: the superfamily of subtilisin-like serine proteases. Protein Science, 6: 501-23.
4. Stahl, M.L. and E. Ferrari, 1984. Replacement of the *Bacillus subtilis* subtilisin structural gene with an in vitro-derived deletion mutation. J. Bacteriol., 158: 411-418.
5. You, L. and F.H. Arnold, 1994. Directed evolution of subtilisin E in *Bacillus subtilis* to enhance total activity in aqueous dimethylformamide. Protein Engineering, 9: 77-83.
6. Kuchner, O. and F. Arnold, 1997. Directed evolution of enzyme catalysts. Trends in Biotechnol., 15: 523-30.
7. Bryan, P.N., M.L. Rollonce, M.W. Pantoliano, J. Wood, B.C. Finzel, G.L. Gilliland, A.J. Howard and T.L. Poulas, 1986. Proteases of enhanced stability: characterization of a thermostable variant of subtilisin. Proteins Structure Function Genetics, 1: 326-334.
8. Liao, H., T. McKenzie and R. Hageman, 1986. Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. Proceedings of the National Academy of Sciences. USA., 83: 576-580.
9. Joyet, P., N. Declerck and C. Gaillardin, 1992. Hyperthermostable variants of a highly thermostable alpha-amylase. Bio/Technol., 10: 1579-1583.
10. Zhao, H. and F.H. Arnold, 1999. Directed evolution converts subtilisin E into a functional equivalent of thermitase. Protein Engineering, 12: 47-53.
11. Cunningham, B.C. and J.A. Wells, 1987. Improvement in the alkaline stability of subtilisin using an efficient random mutagenesis and screening procedure. Protein Engineering, 1: 319-325.
12. Oliphant, A.R. and K. Struhl, 1989. An efficient method for generating proteins with altered enzymatic properties: application to beta-lactamase. Proc. Natl. Acad. Sci. USA., 86: 9094-9098.
13. Chen, K. and F.H. Arnold, 1991. Enzyme engineering for nonaqueous solvents: random mutagenesis to enhance activity of subtilisin E in polar organic media. Bio/Technol., 9: 1073-1077.
14. Chen, K. and F.H. Arnold, 1993. Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide. Proceedings of the National Academy of Sciences. USA., 90: 5618-5622.
15. Miller, J.H., 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.
16. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.
17. Boylan, R.J., N.H. Mendelson, D. Brooks and F.E. Young, 1972. Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid. J. Bacteriol., 110: 281-290.

18. Cutting, S.M. and P.B. Vander Horn 1990. In Harwood, C.R. and Cutting, S.M. (Eds). Molecular biological methods for *Bacillus*: Genetic Analysis. John Wiley and Sons, Sussex, England, pp: 27-24.
19. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
20. Lowry, O.H., A.L. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.
21. Nakamura, T., Y. Yamagata and E. Ichishima, 1992. Nucleotide sequence of the subtilisin NAT gene, aprN, of *Bacillus subtilis* (natto). Bioscience Biotechnol. Biochem., 56: 1869-1871.
22. Smith, E.L., R.J. DeLange, W.H. Evans, M. Landen and F.S. Markland, 1968. Subtilisin Carlsberg. V. The complete sequence; comparison with subtilisin BPN'; evolutionary relationships. J. Biol. Chem., 243: 2184-2191.
23. Schnell, N., G. Engelke, J. Austin, R. Rosenstein, V. Ungermann, F. Gotz and K.D. Entian, 1992. Analysis of genes involved in the biosynthesis of lantibiotic epidermin. European J. Biochem., 204: 57-68.
24. Chen, C.C. and P.P. Cleary, 1990. Complete nucleotide sequence of the streptococcal C5a peptidase gene of *Streptococcus pyogenes*. J. Biol. Chem., 265: 3161-3167
25. Kano, H., S. Taguchi and H. Momose, 1997. Cold adaptation of a mesophilic serine protease, subtilisin, by *in vitro* random mutagenesis. Applied Microbiol. Biotechnol., 47: 46-51.
26. Jain, S.C., U. Shinde, Y. Li, M. Inouye, H.M. Berman, 1998. The crystal structure of an autoprocessed Ser_{221}Cys-subtilisin E-propeptide complex at 2.0-A resolution. J. Molecular Biol., 287: 137-134.
27. Markland, F.S. and E.L. Smith, 1967. Subtilisin BPN. VII. Isolation of cyanogen bromide peptides and the complete amino acid sequence. J. Biol. Chem., 242: 5198-5211.