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Anti-inflammatory activity of a serine protease produced from Bacillus pumilus SG2

Ragupathy Sangeetha[a,*], Irulandi Arulpandi[b]

[a] Department of Biochemistry, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies, Chennai, India
[b] PG and Research Department of Microbiology, Asan Memorial College of Arts and Science, Chennai, India

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ABSTRACT

Proteases have appreciable anti-inflammatory activity and proteolytic enzymes from diverse sources have been studied for their anti-inflammatory potential. This study investigated the anti-inflammatory activity of a protease isolated from *Bacillus pumilus* SG2 using in-vitro models such as heat and hypotonicity induced hemolysis and protein denaturation. The activity exhibited by SG2 protease was comparable to that exhibited by the standard drug diclofenac. The IC 50 value of SG2 protease for inhibition of heat induced hemolysis was calculated to be 226 µg while that of diclofenac was 215 µg The IC 50 value for both protease and diclofenac for the inhibition of hypotonicity induced hemolysis was 85 µg. The IC 50 value of SG2 protease for the inhibition of protein denaturation was 247 µg while that of diclofenac was 181 µg. The structure of SG2 protease was deduced using online tools. The enzyme had a signal peptide of 31 amino acids and a pro-peptide of 77 amino acids. The mature protein consisted of 298 amino acids. The catalytic triad, oxyanion hole and secondary structure of SG2 protease was also studied. Thus a protease with anti-inflammatory potential was studied and was structurally characterized, the details of which may help in engineering the enzyme.

1. Introduction

Proteases are hydrolytic enzymes ubiquitous in nature with both physiological and commercial significance. Proteolytic enzymes have medicinal uses and several plant and microbial proteases have been isolated and their medicinal value has been evaluated. Proteases have developed as effective therapeutic agents (Kim et al., 2006).

Oral administration of proteases from *Aspergillus oryzae* (Luizym and Nortase) has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes (Mikawlrawng, 2016). Clostridial collagenase or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds (Riley and Herman, 2005). Proteases which can catalyse fibrinolysis have been reported (Kim et al., 2006). Proteases, both plant and microbial, have anti-inflammatory potential. Proteolytic enzymes are effective denture and contact lens cleansers. These enzymes are also used to treat necrosis, cancer and cardiovascular disorders (Hellgren et al., 1986; Chanalia et al., 2011).

Proteases are reportedly potential anti-inflammatory drugs. They have been proved to act independently or synergistically with non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are commonly used to treat inflammation. Nevertheless, the side effects of the use of NSAIDs are adverse and hence use of bioactives and bioenzymes with anti-inflammatory activity will help in decreasing the usage of NSAIDs (Swamy and Patil, 2008, Chanalia et al., 2011). Microbes are preferred to plants and animals as sources of proteases because they are generally cheaper to produce, their enzyme contents are more predictable and controllable, reliable supplies of raw material of constant composition are more easily arranged, and plant and animal tissues contain more potentially harmful materials than microbes, including phenolic compounds, endogenous enzyme inhibitors etc (www.lsbu.ac.uk/biology/enztech/sources). Microbes have undermined plants and animals as sources of enzymes due to their broad biochemical diversity, ease of mass culture and also to the ease with which they can be genetically modified (Ishwarya and Sangeetha, 2013).

We had earlier reported the production and purification of a protease from *Bacillus pumilus* (Sangeetha et al., 2010). In the present study we have analysed the secondary structure of protease and investigated the anti-inflammatory potential of *Bacillus pumilus* protease using in vitro models.

* Corresponding author.
E-mail address: sara_dna@yahoo.co.in (R. Sangeetha).

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2. Materials and methods

2.1. Enzyme production

A promising strain *Bacillus pumilus* SG2 which produced protease was isolated and maintained on agar slants at 4 °C. The production medium consisted of (w/v) 0.04% CaCl2, 0.02% MgCl2, 1% glucose, 0.5% NaCl and 0.3% yeast extract (in sodium phosphate buffer, pH 9.0). Five ml of overnight culture (O.D600 = 1.0) of *Bacillus pumilus* SG2 was inoculated into 100 ml production medium and incubated on a rotary shaker (180 rpm) for 48 h at 37°C. At the end of the incubation period, the production medium was harvested and centrifuged. The supernatant was collected and protease present in it was assayed by the method of Rahman et al., 2005. One unit (U) of protease activity is equivalent to 0.5 µg of tyrosine liberated by 1.0 ml of enzyme solution under the assay conditions.

The culture supernatent was subjected to purification by ammonium sulphate fractionation, dialysis and Sephadex G-75 gel filtration chromatography, as reported earlier (Sangeetha et al., 2010). The protease fraction (SG2 protease) was collected and used for further studies.

2.2. Models for Invitro anti-inflammatory activity

Stabilization of human red cell membrane by the enzyme SG2 protease was studied using heat and hypotonicity induced hemolysis methods. Blood was collected from a healthy who had not taken any NSAID for 2 weeks prior to the experiment. It was mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline (0.9%) and a 10% human red blood cell (HRBC) suspension was prepared using isosaline.

Hypotonicity induced hemolysis:

The assay was carried out following the method of Gandhisan et al., 1991. To 1mL phosphate buffer (pH 6.5), 2mL hyposaline (0.36%), and 0.5mL of HRBC suspension, various dilutions of the protease preparations were added. These tubes were then incubated for 30 min at 37 °C. The tubes were then centrifuged at 3,000 rpm for 20 min and the hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac (1mg/mL) was used as reference standard and a control was by prepared by omitting the enzyme. The results were expressed as percent inhibition of hemolysis when compared to control. The experiments were performed as triplicates.

Heat induced hemolysis

To 1mL phosphate buffer (pH 6.5) and 0.5mL of HRBC suspension, various dilutions of the protease preparations were added. The tubes were kept in a boiling water bath at 60 °C for 30 min. The tubes were cooled at the end of the incubation period, centrifuged at 3,000 rpm for 5 min and the hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac (1mg/mL) was used as reference standard and a control was by prepared by omitting the enzyme. The results were expressed as percent inhibition of hemolysis when compared to control. The experiments were performed as triplicates.

Inhibition of denaturation of albumin

This activity was studied by the method of Heendeniya et al., 2018. To 1 mL phosphate buffer (pH 6.5) and 200 µl of egg albumin (from hen egg) various dilutions of the protease preparations were added. The tubes were kept in a boiling water bath at 40 °C for 15 min, the temperature of the water bath was increased gradually to 70 °C and incubation was done for 5 min. The tubes were cooled at the end of the incubation period and the contents were read spectrophotometrically at 660 nm. Diclofenac (1mg/mL) was used as reference standard and a control was by prepared by omitting the enzyme. The results were expressed as percent inhibition of hemolysis when compared to control. The experiments were performed as triplicates.

The percentage inhibition of hemolysis/denaturation was calculated using the following formula:

Percentage inhibition = (1 - Absorbance of sample/ Absorbance of control) X 100

2.3. Statistical analysis

Results are expressed as Mean ± SD of three independent experiments. The difference in activity exhibited by different concentrations of protease groups was compared by One-Way Analysis Of Variance (ANOVA) followed by Dunnet Multiple comparison test (control Vs test).

3. Results and Discussion

Inflammation is a reaction process invoked by several physical and chemical agents, infections and diseases. The process of inflammation is manifested as heat, redness, edema and pain, all of these caused primarily by the damage to tissue proteins and release of lysosomal enzymes. Hence, inhibition of protein denaturation and stabilisation of lysosomal membranes may prevent the onset of inflammation. The efficacy to inhibit protein denaturation and membrane lysis will apparently indicate anti-inflammatory potential. The anti-inflammatory potential of the protease from *B. pumilus* SG2 was analysed with hemolysis and protein denaturation models.

The inhibitory effect of the enzyme SG2 protease was studied at various concentrations (40 – 1280 µg) and was compared with the standard anti-inflammatory drug namely diclofenac. The inhibition effect was found to increase steadily with increase in concentrations of the enzyme. The concentration of SG2 protease required to achieve 50% inhibition of hemolysis and denaturation was 320 µg. The IC 50 value of protease for inhibition of heat induced hemolysis was calculated to be 226 µg while that of diclofenac was 215 µg (Figure 1). Thus the effect of SG2 protease was almost comparable to that of diclofenac. The IC 50 value for both protease and diclofenac for the inhibition of hypotonicity induced hemolysis was 85 µg (Figure 2) and this indicates that protease is as effective as diclofenac in exhibiting anti-inflammatory effect. The lysosomal enzymes released during inflammation produce a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The NSAIDs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC is similar to lysosomal

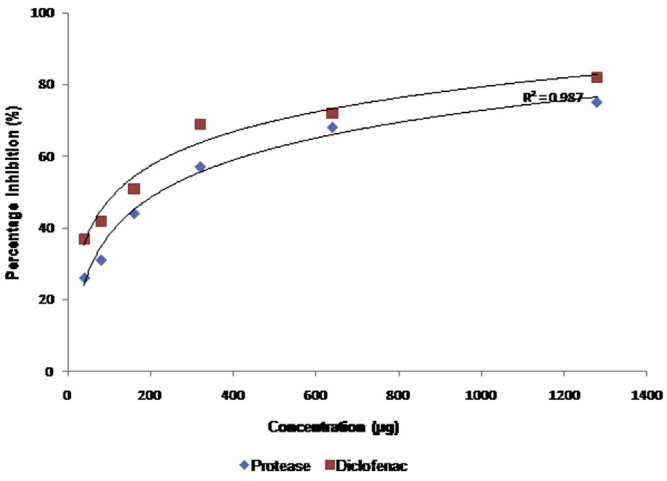

Figure 1. Caption: Inhibition of heat induced hemolysis by SG2 protease. Footnote: All the results given are Mean of three independent experiments.

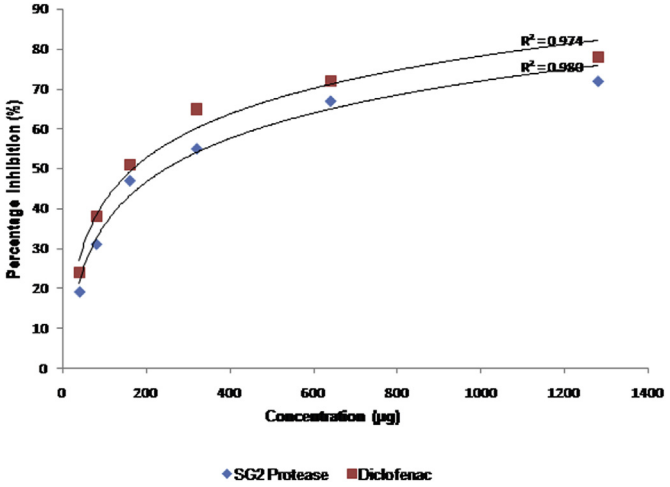

Figure 2. Caption: Inhibition of hypotonicity induced hemolysis by SG2 protease. Footnote: All the results given are Mean of three independent experiments.

membrane components, the prevention of hypotonicity-induced HRBC membrane lysis is taken as a measure of anti-inflammatory activity of the drugs.

The IC 50 value of SG2 protease for the inhibition of protein denaturation was 247 µg while that of diclofenac was 181 µg (Figure 3). However, the concentration of 1280 µg of protease and diclofenac exhibited 76 ± 2.3% and 80 ± 3.1% inhibition respectively and thus the efficacy of protease was comparable to that of diclofenac. Denaturation of tissue proteins triggers the formation of antigens thereby initiating type III hypersensitivity which culminates in inflammation and thus any agent which could cause the inhibition of protein denaturation will have promising therapeutic potential as an anti-inflammatory agent (Agrawal and Paridhavi, 2007).

An understanding of the amino acid sequence of the enzyme is essential to interpret the catalytic efficacy of the enzyme and for enzyme engineering as well. Directed evolution of enzymes is being explored worldwide so as to produce recombinant enzymes with enhanced catalytic efficacy. Hence the primary and secondary structure of SG2 protease was analysed using online tools. The coding sequence of the serine protease produced by *B. pumilus* SG2 was deduced and reported (Sangeetha et al., 2019). SignalP, version 3.0 was used to predict the signal peptide sequence of SG2 protease (Figure 4). A signal peptide of 31 amino acids (M1 – A31) and a pro-peptide of 77 amino acids (E32-Y77) were identified. The mature protein consisted of 298 amino acids.

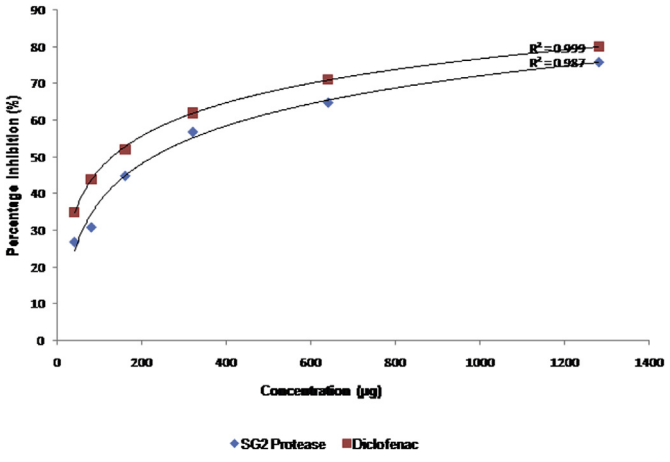

Figure 3. Caption: Inhibition of protein denaturation by SG2 protease. Footnote: All the results given are Mean of three independent experiments.

The cleavage of sites of pre and pro-peptides were conserved among other *Bacillus* subtilisins. Thus like many other proteases, SG2 protease is synthesized as inactive pro-enzymes. The pro-region is generally believed to function as intramolecular chaperone, guiding the corrected folding of the protease domain and preventing it from being active until the pro-region is removed in the appropriate compartment (Rahman et al., 2007; Jaouadi et al., 2008).

The catalytic triad composed of the three highly conserved residues Aspartate (D) 32, Histidine (H) 64 and Serine (S) 221 was found in the SG2 protease. Thus SG2 protease belongs to the subtilisin clan of proteases (). Serine proteases have been classified into evolutionarily unrelated clans, which have been subdivided into families of proteases whose homology can be established statistically (Rawlings and Barrett, 1993; Barrett and Rawlings, 1995). Clans differ in terms of overall fold and the order of catalytic residues in the primary sequence. Despite these significant differences, serine proteases of clans SA (chymotrypsin-like), SB (subtilisin-like) and SC (α/β-hydrolase fold) maintain strictly conserved active site geometry among their catalytic Ser, His and Asp residues. Enzymes of clan SB have been reported to use exclusively TCN codon for the active site serine (Rawlings and Barrett, 1994). This could be observed in SG2 protease also. The sequence motifs surrounding the active site residues and evolutionary markers show that SG2 protease belongs to S8C subfamily of SB clan (Krem and Di Cera, 2001). The serine proteases are known to have an active site consensus sequence G-X-S-X-A (Gupta et al., 2002) and this was present around the active site serine 221 in SG2 protease.

The oxyanion-hole asparagine was conserved in SG2 protease at the position 155 as seen in other subtilisin-like proteases. The catalytic mechanism of serine proteases proceeds via the formation of one or more tetrahedral intermediates. In the serine proteases, two peptide NH groups of the polypeptide backbone form the so-called oxyanion hole by donating hydrogen bonds to the negatively charged oxygen atom of the tetrahedral intermediate. In the bacterial protease, subtilisin the side chain of Asn-155 is believed to provide one of the hydrogen-bonding groups in the oxyanion hole. The oxyanion-hole asparagine155 is highly conserved in all subtilisn-like proteases. In subtilisn, the amide of the catalytic serine residue (Ser 221) in an oxyanion hole hydrogen bond donor and a second interaction is contributed by the amide side chain of asparagine155 (Bryan et al., 1986).

Glycine residues G142, G173, G191, G262, and G327 (Stormo et al., 1982) that were highly conserved in most of subtilases were totally conserved in SG2 protease. ProtParam tool at http://www.au.expasy.org/cgi-bin was used to determine the amino acid composition of SG2 protease. 15 residues were negatively charged (Asp + Glu), while 20 residues were positively charged (Arg + Lys + His). The total charged residues (Asp, Glu, Arg, Lys, His) were 35 amino acids or 11.7% of the total amino acids. The hydrophobic amino acids Ala, Ile, Leu, Met, Phe, Pro, Trp and Val make up to 130 amino acids (43.6%) and the total uncharged residues (Asn, Cys, Gln, Gly, Ser, Thr, and Tyr) were 133 amino acids (44.6%). The instability index of SG2 protease was computed to be 23.15 and hence the protein is stable. Enzymes are considered as unstable when the instability index is higher than 40 (Rahman et al., 2007). The most significant feature of the amino acid composition of SG2 protease was its Asx (Asp+Asn) content. SG2 protease contains 35 Asx residues (24Asn + 11Asp) and thus its content (11.7%) was higher than that of Subtilisin BPN' and Subtilisin Carlsberg.

The calculated molecular weight of the mature form of SG2 protease was 30.5 kDa. The experimental molecular weight calculated based on SDS-PAGE analysis was 28 kDa (Sangeetha et al., 2010). This difference in the molecular weight of the enzyme could be attributed to the post-translational processing of the protease after being secreted extracellularly.

Predict protein server was used to study the primary and secondary structure details of SG2 protease. PSI-BLAST multiple sequence alignment showed that SG2 protease was highly similar to many serine

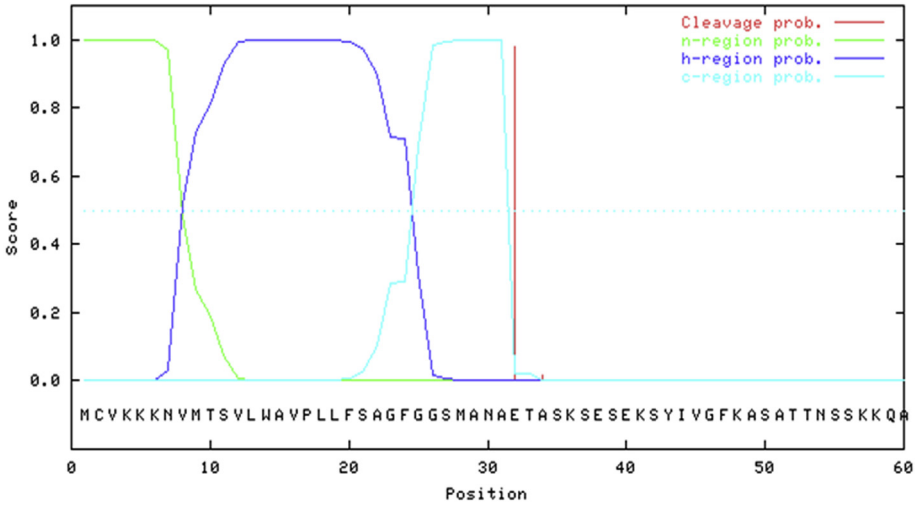

Figure 4. Caption: Signal peptide and pro-peptide analysis of SG2 protease. Footnote: Signal P, version 3.0 was used to predict the cleavage regions of SG2 protease

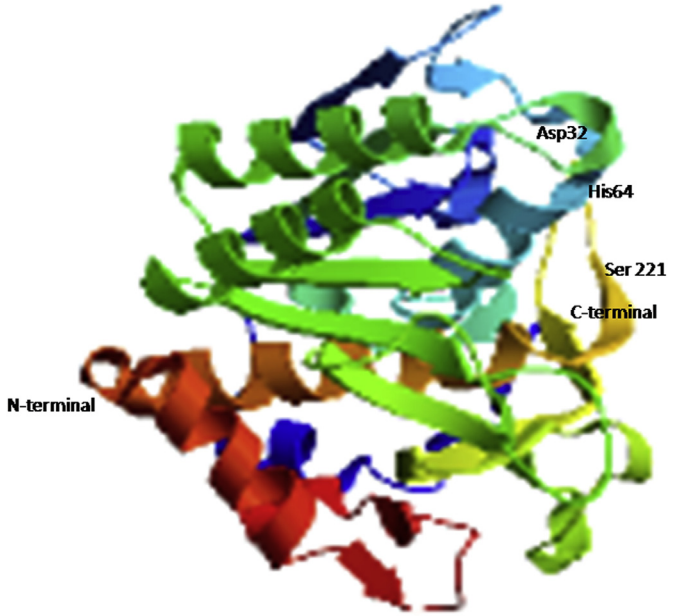

Figure 5. Caption: Proposed 3 D structure of SG2 protease. Footnote: The 3D model of the enzyme was deduced using the SWISS-MODEL server. The amino acid residues 1- 274 were chosen for model prediction

proteases of *Bacillus* species. PROF predictions revealed that the secondary structure of SG2 protease contained 19.8% alpha helices and 20.13% extended beta sheets. The remaining 60% of the protein was present as loops. Thus SG2 protease was classified as a mixed protein. The solvent accessibility composition of SG2 protease was also predicted using PROF predictions. Nearly 55% of the amino acids of SG2 protease had 16% of their surface exposed and were accessible to solvents. Alpha helices were predicted in the following regions: between amino acids 15-18, 103-115, 133-143, 224-236, 243-253 and 270-278. Extended beta sheets were predicted between amino acids 27-32, 67-74, 80-85, 89-96, 121-124, 147-153, 173-180, 196-199, 204-207 and 266-268. PROSITE motif search was used to predict the sites of post-translational modifications. Glycosylation was predicted at asparagine residues at positions 56, 123, 130, 187 and 240. Tyrosine kinase and casein kinase phosphorylation sites were predicted at Arg 98 and Ser 109. N-myristoylation of glycine residues were predicted at positions 35, 64, 83, 110, 127, 154, 160 and 219.

The theoretical 3D structure of the enzyme was deduced using the SWISS-MODEL workspace. The 298 amino acid residues of SG2 protease were submitted and 1- 274 amino acid residues were considered by the server primarily based on the alignment of SG2 protease with the template '1scjA' and the structure predicted using '1scjA' is given Figure 5. The biochemical characterization studies proved that SG2 protease was not metal-dependent (Sangeetha et al., 2010). This was reassured by the absence of the metalloprotease metal binding motif, HEXXH.

4. Conclusion

A protease was produced from *Bacillus pumilus* SG2 and was studied for its anti-inflammatory potential. The enzyme was able to inhibit heat and hypotonicity induced hemolysis and protein denaturation. The efficacy to inhibit hemolysis and denaturation proved that the enzyme has anti-inflammatory potential and the IC 50 values observed with the inhibition studies implied that the enzyme SG2 protease is as potent as the standard drug diclofenac. The secondary structure of SG2 protease was also characterized as the knowledge of an enzyme's secondary structure will be useful to engineer the enzyme and increase its therapeutic efficacy.

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