Malaysian Journal of Microbiology, Vol 14(6) Special Issue 2018, pp. 452-461
DOI: http://dx.doi.org/10.21161/mjm.1461802

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Gene isolation and prediction of the corresponding three-dimensional structure of subtilisin from the psychrophilic yeast, *Glaciozyma antarctica* PI12

Siti Mardhiah Mustafha[1], Shazilah Kamaruddin[1], Nor Muhammad Mahadi[2,3], Abdul Munir Abdul Murad[1], Farah Diba Abu Bakar[1]*

[1]School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[2]Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[3]Malaysian Genome Institute, Jalan Bangi Lama, 43000 Kajang, Selangor, Malaysia.
Email: fabyff@ukm.edu.my

ABSTRACT

Aims: Subtilisin, a serine protease, is a key player in many industrial applications especially in the detergent industry. Most reported subtilisins originate from mesophilic and thermophilic microorganisms. Only scarce information about cold-active subtilisins from psychrophilic microbes is available. Here we describe the isolation, cloning and *in silico* characterisation of a gene encoding subtilisin in the obligate psychrophilic yeast, *Glaciozyma antarctica* PI12.
Methodology and results: A full-length cDNA from *Glaciozyma antarctica* encoding subtilisin (*GaSUB*) was isolated through Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) techniques. The open reading frame of *GaSUB* comprised 1,125 nucleotides encoding 375 amino acids. The *Ga*SUB amino acid sequence had 49% sequence identity with a subtilisin from the yeast, *Puccinia striiformis*. Bioinformatic analyses revealed that the *Ga*SUB protein contains a domain that represents the S8 domain of the largest protease family. The predicted model of *Ga*SUB protein using MODELLER and Pymol software revealed that this enzyme has longer loops and less intramolecular interactions between amino acid residues as compared to its mesophilic and thermophilic counterparts. These characteristics are known to help in protein flexibility and stability in cold-active enzymes.
Conclusion, significance and impact of study: Bioinformatics characterisations suggested that this enzyme is uniquely adapted to cold environments. Further work using amplified cDNA will be conducted to confirm the catalytic function of this enzyme.

Keywords: S8 protease family, cold-active enzyme, protein homology modelling

INTRODUCTION

Almost 85% of Earth's surface is constantly exposed to temperature less than 5 °C however, this vast area contains a wide variety of psychrophilic organisms including algae, filamentous fungi, archaea, protozoa and yeasts (Buzzini *et al*., 2012). Psychrophilic organisms are capable of striving in cold conditions due to several adaptations including modification of membrane compositions, production of compatible solutes and secretion of cold active enzymes (D'Amico *et al*., 2006). Proteases, in particular serine proteases, can easily be found in various organisms such as bacteria, fungi and eukaryotes (Sabotic and Kos, 2012). Serine proteases are popularly used in numerous industrial applications such as in the detergent industry, leather tanning, silver recovery, food industry and waste treatment (Jisha *et al*., 2013). Cold active enzymes including serine proteases have gained attention as an alternative for mesophilic enzymes in many industrial processes (Sarmiento *et al*., 2015) due to their high catalytic activity at low temperature, thermolability at high temperature and wide range of substrate specificity (Kasana, 2010). The high catalytic activity of cold active enzymes at low temperatures allows for a small amount of enzymes for catalysis. Thus, this indirectly reduces the cost of enzyme preparation whilst their heat lability will allow these enzymes to be inactivated when moderate heat is being introduced (Feller, 2013).

The subtilisin family (also known as the S8 family), is the second largest family of serine proteases (Rawlings *et al*., 2009). Due to their industrial importance, subtilisins are amongst the best studied enzymes (Gupta *et al.*, 2002; Ran *et al*., 2014). Proteases, especially subtilisins, have been used for more than fifty years in the detergent industry. Subtilisins added to modern laundry detergents help boost detergent performance by removing proteinaceous deposits (Vojcic *et al*., 2015). Subtilisins have been identified in many organisms but most of those

***Corresponding author**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

used in detergents have been isolated from *Bacillus* species (Lylloff *et al*., 2016).

Subtilisins are also used in food processing as well as in the leather and textile industries that requires high temperatures and heat-stable enzymes. High temperatures and harsh reaction conditions indirectly lead to environmental pollution and safety hazards (Gupta *et al.*, 2002). Industrial enzymes with high catalytic activities at low temperatures could provide an economic and environmental alternative which would reduce energy consumption as well as protect the texture and colours of fabrics (Struvay and Feller, 2012). Furthermore, subtilisins of fungi and/or yeasts are easier to isolate and purify (Kumar *et al*., 2008). Here we described the isolation, cloning, sequence analysis and protein homology modelling of a putative subtilisin from the psychrophilic *G. antarctica* PI12. Bioinformatic analyses showed that the *G. antarctica* subtilisin *Ga*SUB possesses psychrophilic properties. Ultimately, we hope to produce a recombinant cold-active subtilisin enzyme of industrial interest.

MATERIALS AND METHODS

Strains

The *G. antarctica* PI12 psychrophilic yeast was isolated from the Casey Research Station, Antarctica (Hashim *et al*., 2013). A medium containing yeast extract, peptone and dextrose (YPD) supplemented with 50 µL/mL kanamycin and 50 µL/mL ampicillin was used to culture PI12 for seven days at 12 °C and 200 rpm rotation. *Escherichia coli* DH5α and BL21 strains were obtained from the Culture Collection of the Malaysia Genome Institute (MGI).

Isolation and cloning of the full-length subtilisin cDNA

TRIzol™ reagent (Invitrogen by Life Technologies/Thermo Fisher Scientific Waltham, MA USA) was employed in the extraction of total RNA from PI12 cultures using the protocol of manufacturer. Extracted total RNA was used as a template for reverse transcription to generate first-strand cDNA. RT-PCR was performed using Superscript® III First-Strand Synthesis System kit (Invitrogen by Life Technologies). The total volume of reverse transcription reaction mixture was 20 µL and comprised of 1.5 µg total RNA, 200 U Superscript® III RT, 50 µM oligo(dT)20, RT buffer and 2 × cDNA synthesis reaction mix. The reaction was initiated by incubation at 65 °C for 5 min, followed by 50 °C for 50 min. The reaction was then terminated at 85 °C for 5 min. The cDNA reaction product was used directly for PCR. The full-length subtilisin cDNA fragment was amplified using 1 µL of template cDNA, 1 U KOD Hot Start DNA polymerase, 10 × KOD Hot Start DNA polymerase buffer, 25 mM $MgSO_4$, 2 mM dNTPs, 10 µM of *GaSUB*_F (5'-GACGACGACAAGATGGGCGCCAGCTCCT-3') and *GaSUB*_R (5'-GAGGAGAAGCCCGGTCACGAGCGGA-3') specific primers. PCR was carried out initially using denaturation at 95 °C for 2 min, succeeded by denaturation at 30 cycles, 95 °C for 20 sec, annealing at 63 °C for 10 sec, extension at 70 °C for 20 sec and finally, an extension at 70 °C for 5 min. The amplicons were then analysed on an agarose gel (1%) electrophoresis before cloning into the vector, pGEM®-T Easy Vector System (Promega, Madison, WI, USA), followed by transformation of DH5α *E. coli.* Universal primers were used to sequence selected plasmids carrying the target *GaSUB* (First BASE Laboratories, Seri Kembangan, Malaysia) to confirm fidelity.

Bioinformatic analyses

The cloned subtilisin gene was aligned against other subtilisin genes in the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using BLASTp (Basic Local Alignment Search Tool) programme (http://blast.ncbi.nlm.nih.gov/) (Altschul *et al*., 1997). The functional amino acid sequence, theoretical iso-electric point and molecular mass were determined using ExPASy tools (http://www.Expasy/tools) (Artimo *et al*., 2012). The SignalP 4.1 Server was used to detect the presence of a signal peptide (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al*., 2011). The identification of important subtilisin domains was performed by InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) (Quevillon *et al*., 2005). Multiple sequence alignment of the *Ga*SUB amino acid sequence was conducted using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Larkin *et al*., 2007) and the BoxShade server (http://www.ch.embnet.org/Software/BOX_form.html). Protein structure prediction was performed using MODELLER version 9.18 software (https://salilab.org/modeller/) (Webb and Sali, 2014) and the structure was viewed by Pymol version 1.8.6.2 software (https://www.pymol.org/) using the subtilisin structure from *Bacillus subtilis* (Protein Data Bank/PDB ID: 3WHI) as template. The predicted protein structure was validated and analysed using the Ramachandran plot server, RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) (Wang *et al*., 2016). The structure comparison was analysed using the PDBeFold server (http://www.ebi.ac.uk/msd-srv/ssm/) (Krissinel and Henrick, 2004). The protein Interaction Calculator (PIC) server (http://pic.mbu.iisc.ernet.in/) (Tina *et al.*, 2007) was used to calculate and predict the total number of various protein interactions within the predicted protein structure. The pocket (cavity) numbers of the predicted structure was performed using POCASA server (http://altair.sci.hokudai.ac.jp/g6/service/pocasa/) (Yu *et al*., 2010). Exposed surfaced area was calculated using VADAR software (https://vadar.wishartlab.com) (Willard *et al.*, 2003).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 452-461
DOI: http://dx.doi.org/10.21161/mjm.1461802

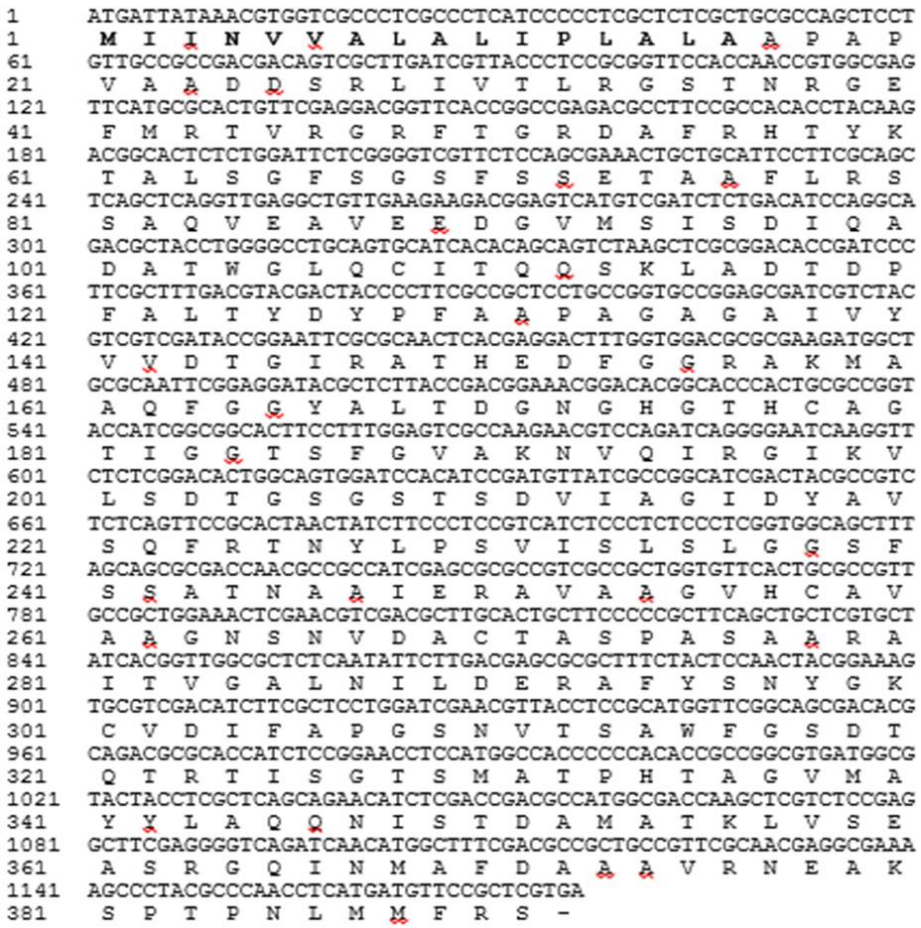


Figure 1: The *Glaciozyma antarctica* PI12 *GaSUB* cDNA (1,176 bp full-length sequence) sequence encoding a subtilisin. Capital letters in black represent nucleotides whilst grey capital letters are the encoded amino acid sequence. The putative signal peptide is represented by sixteen amino acid residues (bold letters) starting from the methionine (M) residue. The 'ATG' and 'TGA' (boxed and shaded) respectively designate the translational start and stop codons.

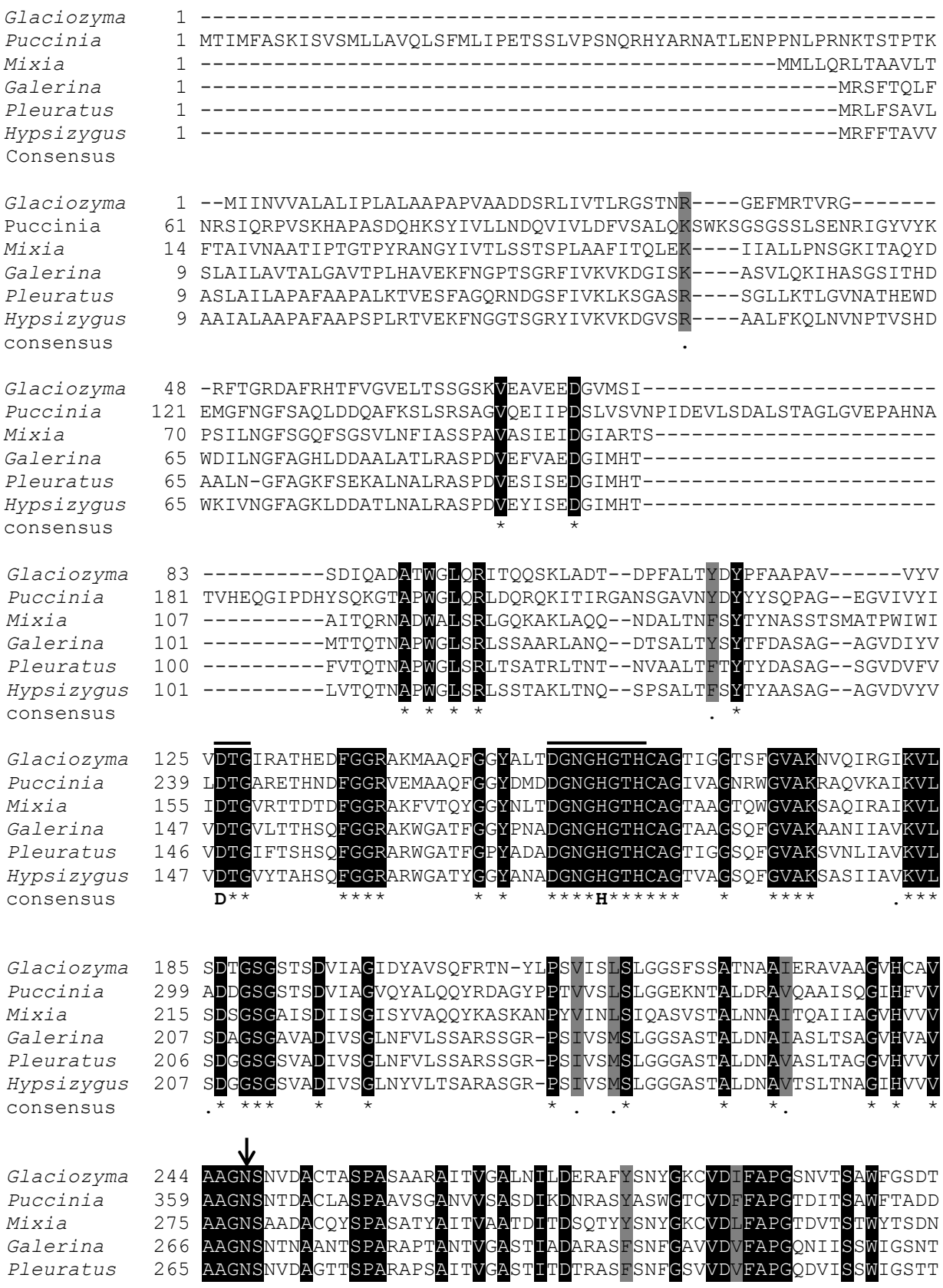
RESULTS AND DISCUSSION

Cloning and sequence analyses

Total RNA was obtained from 7-day old cultures of *G. antarctica* PI12. First-strand cDNA was generated by RT-PCR with the template from total RNA. The full-length cDNA encoding *GaSUB* was generated using the specific primers *GaSUB*_F and *GaSUB*_R. Sequence analyses revealed that the full length cDNA of *GaSUB* is 1,176 bp sequence (Figure 1) where 48 bp of the 5' upstream sequence is the secretion signal that encodes a signal peptide which facilitates the secretion of this protein to the extracellular medium (Hashim *et al*., 2013). The deduced amino acid sequence contained 375 amino acids (Figure 1) with a computed molecular mass of 38.9 kDa and a predicted iso-electric point (pI) of 6.07. InterProScan revealed that *Ga*SUB has a subtilisin S8 family domain, the main domain of the subtilisin family within the subclass, serine proteases. The subclass serine protease is the largest class in the serine protease group (Yang *et al*., 2011). BLASTp searches performed using the deduced amino acid sequence against other subtilisins from different species showed that the highest sequence identity was with the subtilisin of *Puccinia striiformis* (49% identity, e-value of 3e-98) (GenBank Accession KNE95710.1) followed by *P. triticina* (49% identity, 1e-97) (GenBank Accession OAV94465.1) and *Puccinia sorghi* (51% identity, 7e-96) (GenBank Accession KNZ44180.1). The motifs, DG-GHGTH, DTG and GTSMA-PH are highly conserved amongst fungal proteases (Li and Li, 2009) (Figure 2). These motifs are made up of aspartic acid (D), histidine (H) and serine (S) that form the catalytic triad (Asp/His/Ser - from the N to C terminus) which is a common feature of most subtilisin S8 family members (Ekici *et al*., 2008).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 452-461
DOI: http://dx.doi.org/10.21161/mjm.1461802



 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 452-461
DOI: http://dx.doi.org/10.21161/mjm.1461802

Figure 2: Amino acid sequence alignment of subtilisins from: *Glaciozyma antarctica* PI12 (GenBank accession no. MH033855), *Puccinia striiformis* (GenBank accession no. KNE95710.1), *Mixia osmundae* (GenBank accession no. XP_014567424.1), *Galerina marginata* (GenBank accession no. KDR86145.1), *Pleuratus ostreatus* (GenBank accession no. ACR25273.1) and *Hypsyzigus marmoreus* (GenBank accession no. KYQ45716.1). Asterisks and full stops respectively designate residues with identity and weak similarity. Residues responsible for formation of the catalytic triad such as aspartic acid (A), histidine (H), and serine (S) are indicated by capital letters. Three highly conserved motifs are underlined at the top. The conserved active residue, asparagine (N) involved in substrate-enzyme catalysis is indicated by an arrow.

The active serine residue acts as a nucleophile that attacks the scissile carbonyl carbon atom of substrates, an attack aided by the imidazole group of histidine that accepts the proton from the nucleophilic hydroxyl (OH) group. This leads to tetrahedral intermediate formation bearing an oxyanion hole that is stabilised by aspartic acid (an electrophilic catalyst), followed by expulsion of the amine group (Polgar, 2005; Ekici *et al*., 2008). Asparagine (N) is the common signature residue in subtilisin-like serine proteases and assists in the formation of the oxyanion hole during substrate catalysis where the side chain amide group of asparagine constitutes the oxyanion binding site (Polgar, 2005; Ekici *et al*., 2008; Farah *et al*., 2015). A phylogenetic tree was generated [using the neighbour-end joining method – 1,000 bootstrap replicates and subtilisin of *Homo sapiens* (AAV67948.1) as the outgroup] to show the evolutionary relationship of *Ga*SUB to other subtilisins (Figure 3). As expected, *G. antarctica* PI12 subtilisin clustered together with other subtilisins from Basidiomycota. However, the *G. antarctica* PI12 subtilisin-like serine protease precursor referred to as CAQ (CAQ76821.1; Alias *et al*., 2014) was grouped with the Bacilli clad at a high evolutionary relationship as they presumably share a high degree of protein sequence. BLASTp analysis between *Ga*SUB and CAQ76821.1 revealed a low sequence identity (29% with 2e-04 e-value and 19% query coverage) although both enzymes originated from the same organism and strain. Moreover, two of the three conserved motifs of fungal proteases (DG-GHGTH and GTSMA-PH) were not conserved despite having conserved catalytic residues (data not shown). An additional domain, PA_C5a_like, protease-associated domain containing C5a peptidase was found in CAQ (this domain is absent in *Ga*SUB). This domain encodes for invasin enzyme that can be found in bacteria especially in *Streptococcus* species (Brown *et al*., 2005).

Predicted structure analyses

The predicted structure of *Ga*SUB showed characteristics of a cold-active enzyme. A subtilisin from the mesophile *Bacillus subtilis* (3WHI) (28% sequence identity to *Ga*SUB) was chosen as the closest homologue for modelling. The predicted structure of *Ga*SUB (Figure 4a) contained 10 parallel β-strands (S3-S4, S6-13), three anti-parallel β-strands (S1-S2, S5) and nine α-helices. The validity of the predicted structure was controlled using RAMPAGE (Wang *et al*., 2016). Validity analyses revealed that 89.5% of the residues were in the most favoured region, 7.3% of residues were in the generously allowed regions and the rest of the residues were positioned in the disallowed region.

Many structural features including shortened and extended loop, low or high numbers of hydrophobic and hydrogen bond interactions, ion pairs, aromatic interactions and cation-π interactions, low or high numbers of glycine, arginine and proline residues affect protein mobility (Feller, 2010). The superposed structure of *Ga*SUB on subtilisins of the mesophilic 3WHI and the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 452-461
DOI: http://dx.doi.org/10.21161/mjm.1461802

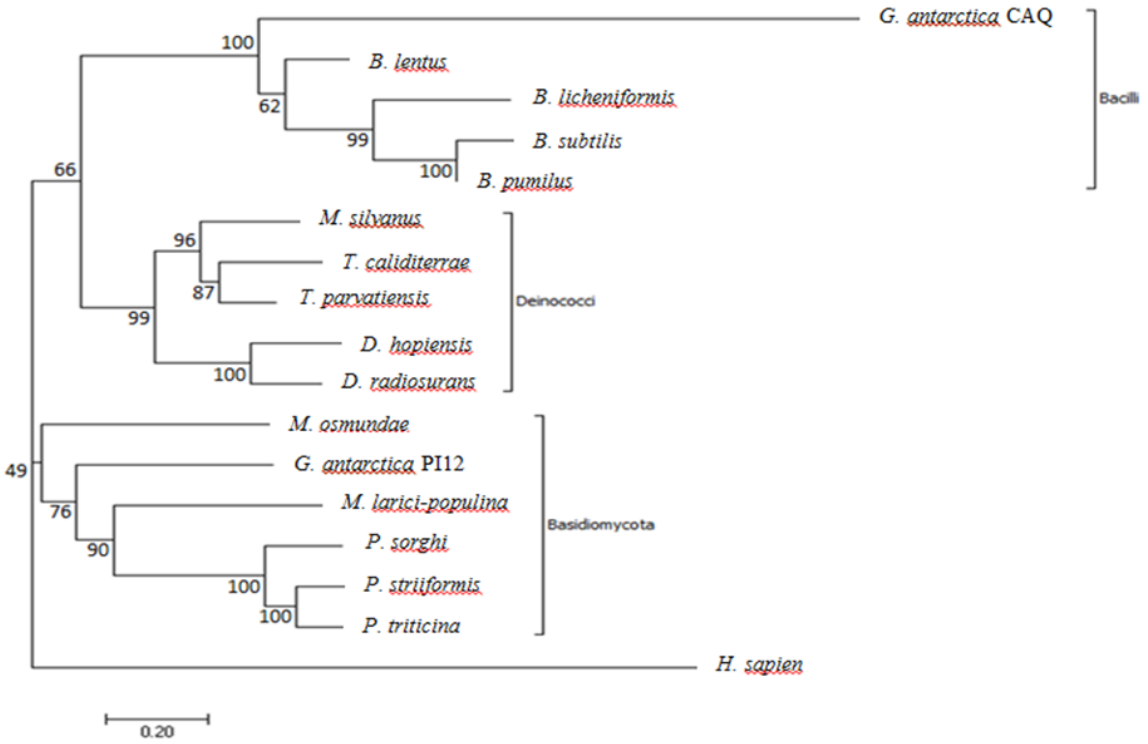


Figure 3: Phylogenetic tree of subtilisins. The tree was constructed using a Neighbor-Joining algorithm with 1000 bootstrappings using MEGA 6 software and the *Homo sapien* subtilisin (AAV67948.1) as the outgroup. Evolutionary distance is shown with the scale of 0.20 % and bootstrap values (%) are designated at the nodes. *Ga*SUB is labelled as *G. antarctica* PI12 (MH033855). Accession numbers of subtilisin sequences from GenBank are as follows: *Glaciozyma antarctica* CAQ (CAQ76821.1), *Bacillus lentus* (P29599.1), *B. licheniformis* (CAA62667.1), *B. subtilis* (CAA74536.1), *B. pumilus* (P07518.1), *Meiothermus silvanus* (WP_013158250.1), *Thermus caliditerrae* (WP_038045160.1), *T. parvatiensis* (EIA39321.1), *Deinococcus hopiensis* (WP_084049387.1), *D. radiodurans* (NP_296043.1), *Mixia osmundae* (XP_014567424.1), *Melampsora larici-poppulina* (XP_007411463.1), *Puccinia sorghi* (KNZ44180.1), *P. striiformis* (KNE95710.1) and *P. triticina* (QAV94465.1).

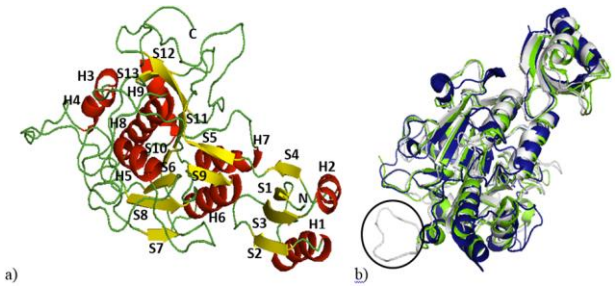


Figure 4: (a) The predicted structure of *Ga*SUB possesses 13 β-strands (S1-S13) and nine α-helices (H1-H9). (b) The superimposed structures between *Ga*SUB (grey), *B. subtilis* subtilisin 3WHI (blue) and *T. kodakarensis* 4JP8 (green) show similar structural architecture; however, an extended loop in the region of Q93 – P114 was observed for *Ga*SUB (circle).

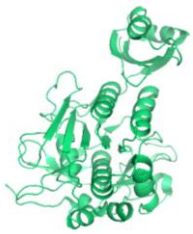
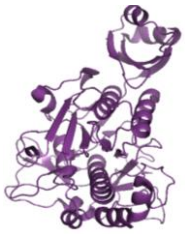
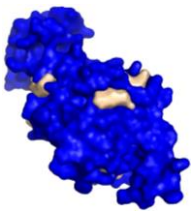
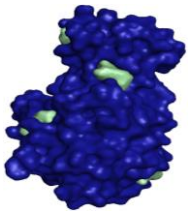
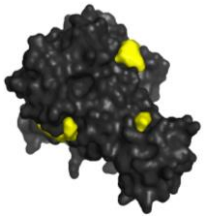
thermophilic *Thermococcus kodakarensis* (4JP8) (Table 1) showed high tertiary structure similarity (96% with rmsd values of 0.821Å and 1.533 Å, respectively) (Webb and Sali, 2014; Krissinel and Henrick, 2004). Nevertheless, superimposition of the *Ga*SUB structure on the template revealed an extended loop on the former (Figure 4b). Loop extension is a general characteristic of psychrophilic enzymes. Longer loops increase the movement of secondary structures that reduce enzyme stability (Pucci and Rooman, 2017; Siddiqui *et al.*, 2006). This observation correlates with the psychrophilic enzyme β-glucosidase from *Micrococcus antarcticus* (Miao *et al*., 2016).

Arginine is more abundant in thermostable enzymes and this contributes to salt bridges and multiple hydrogen bonds. Thermolabile enzymes have a tendency to contain lower numbers of arginine (D'Amico *et al*., 2002). Our data (Table 1) showed that *Ga*SUB has the lowest number of total hydrogen bonds - 628 as compared to 4JP8 (831) and 3WHI (1355) despite having higher arginine content (5.3%) as compared to 3WHI (1.1%) and 4JP8 (2.3%). We postulate that the polar residues such as arginine of *Ga*SUB are involved in interactions on the enzyme surface. An increased number of polar residues on the protein surface area contributes to protein stability

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 452-461
DOI: http://dx.doi.org/10.21161/mjm.1461802

Table 1: Comparison of structural features and interactions between three subtilisins, *Ga*SUB, 3WHI (mesophilic *Bacillus subtilis*) and 4JP8 (thermophilic *Thermococcus kodakarensis*).

	***Ga*SUB**	**3WHI**	**4JP8**
Structure			
Amino acids	374	365	398
Glycine (%)	9.4	11.2	11.3
Proline (%)	3.2	4.4	5.8
Arginine (%)	5.3	1.1	2.3
r.m.s.d		0.821Å for 355 Cα	1.533Å for 395 Cα
Sequence similarity (%)		6.6	7.0
Structure similarity (%)		96	96
Hydrophobic interactions	134	644	399
Hydrogen bonds (MC-MC)	386	798	451
Hydrogen bonds (MC-SC)	138	385	234
Hydrogen bonds (SC-SC)	104	172	146
Ionic interactions	22	42	19
Aromatic-aromatic interactions	3	8	8
Aromatic-sulphur interactions	4	2	0
Cation-pi interactions	6	52	2
Intraprotein disulphide bridges	0	0	0
Pocket (cavity) numbers	4	5	5
			
Exposed surface area (Å)			
Total	16387.5	25393.9	14169.0
Polar	3395.3	7071.9	3352.1
Non-polar	11298.4	15090.8	8543.2

ISSN (print): 1823-8262, ISSN (online): 2231-7538

due to an increase in hydrophilic interactions especially hydrogen bond networks between the molecule and solvent in thermolabile enzymes (Bialkowska *et al*., 2016). This has also been reported by Isaksen *et al*. (2014) for trypsin.

In addition, as expected of a psychrophilic enzyme, *Ga*SUB has the lowest number of hydrophobic interactions (134) as compared to 3WHI (644) and 4JP8 (399) (Table 1). Lower hydrophobic interactions of *Ga*SUB may reflect the lower core hydrophobicity resulting less compactness of inner protein structure (Pucci and Rooman, 2017). Hydrophobic interactions contribute to proper folding but are less stable at lower temperatures. Thus, psychrophilic enzymes tend to minimise hydrophobic interactions (Siddiqui and Cavicchioli, 2006; Struvay and Feller, 2012). However, the non-polar exposed surface area of *Ga*SUB is high (11298.4 Å) compared to the thermophilic 4JP8 (8543.2 Å). Non-polar residues on the surface of proteins hinder hydrogen-bonding interactions of water with the surface, pulling away water molecules to allow more hydrogen bonding with themselves thus enhancing the structure and interactions of water molecules on the protein surface (Raschke, 2006; Siddiqui, 2015). The exposed area of psychrophilic enzymes with a higher amount of hydrophobic (non-polar) amino acids improves the flexibility of proteins. This is because the entropy of water molecules is lowered thus forming ordered structures around non-polar residues (Siddiqui and Cavicchioli, 2006; Feller, 2003).

An increase of glycine content in psychrophilic enzymes may provide local mobility, whilst the reduction in proline content confines backbone rotation especially in loops thus decreasing the rigidity between secondary structures (Feller, 2003; Feller and Gerday, 2003). However, *Ga*SUB has only a small difference in glycine (9.4%) and proline (3.2%) content as compared to 3WHI (Gly 11.2%; Pro 4.4%) and 4JP8 (Gly 11.4%; Pro 5.8%) and this also corresponds to the work of Jaafar *et al*. (2016) on *G. antarctica* fuculose aldolase.

Generally, cold active enzymes have lower residue interactions that contribute to protein structure flexibility within their structures (Feller, 2003; Papaleo *et al*., 2011). Nevertheless, some interactions are similar or higher compared to those of mesophilic or thermophilic enzymes. *Ga*SUB is a little different as compared to thermophilic 4JP8 in terms of ionic interactions (22 and 19) and cation-π interactions (6 and 2) (Table 1). It also has similar aromatic-sulphur interactions as compared to mesophilic 3WHI (4 and 2) (Table 1). Our data and those reviewed by Siddiqui *et al.* (2006) show that psychrophilic enzymes do not necessarily possess all structural features required for flexibility at low temperature. Different proteins adopt various structural features to achieve local or global mobility (Struvay and Feller, 2012). *Ga*SUB is an interesting enzyme for further study since its potential cold active properties may also be manipulated for industrial purposes.

CONCLUSION

GaSUB cDNA was isolated, cloned and characterised *in silico*. Bioinformatics analyses predicted that *Ga*SUB belongs to the subtilisin S8 family and possesses characteristics of psychrophilic enzymes. Expression studies will be carried out to assess the catalytic function of *Ga*SUB.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Science, Technology and Innovation through the research grant 02-05-20-SF0007. The authors would like to thank Prof. William J. Broughton for his suggestions and help in the preparation of this manuscript.

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25, 3389-3402.**

Artimo, P., Joonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., de Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., Grosdidier, A., Hernandez, C., Ioannidis, V., Kuznetsov, D., Liechti, R., Moretti, S., Mostaguir, K., Redaschi, N., Rossier, G., Xenarios, I. and Stockinger, H. (2012). ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Research* **40, 597-603.**

Alias, N., Ahmad Mazian, M., Salleh, A. B., Basri, M. and Raja Abd. Rahman, R. N. Z. (2014). Molecular cloning and optimization for high level expression of cold-adapted serine protease from Antarctic yeast *Glaciozyma antarctica* PI12. *Enzyme Research* **2014, 1-20.**

Bialkowska, A., Gromek, E., Florczak, T., Krysiak, J., Szulczewska, K. and Turkiewicz, M. (2016). Extremophilic proteases: Developments of their special functions, potential resources and biotechnological applications. *In:* Biotechnology of Extremophiles, Grand Challenges in Biology and Biotechnology. Rampelotto, P. H. (ed.). Springer International Publishing, Switzerland. **pp. 300-444.**

Brown, C. K., Gu, Z. Y., Matsuka, Y. V., Purushothaman, S. S., Winter, L. A., Cleary, P. P., Olmsted, S. B., Ohlendorf, D. H. and Earhart, C. A. (2005). Structure of the *streptococcal* cell wall C5a peptidase. *Proceedings of the National Academy of Sciences of the United States* **102(51), 18391-18396.**

Buzzini, P., Branda, E., Goretti, M. and Turchetti, B. (2012). Psychrophilic yeasts from worldwide glacial habitats: Diversity, adaptation strategies and biotechnological potential. *Federation of European Microbiological Societies* **82, 217-241.**

D'Amico, S., Claverie, P., Collins, T., Geolette, D., Gratia, E., Hoyoux, A., Meuwis, M. A., Feller, G. and Gerday, C. (2002). Molecular basis of cold

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

adaptation. *Philosophical Transactions of the Royal Society of London Series B* **357, 917-925.**

D'Amico, S., Collins, T., Marx, J. C., Feller, G. and Gerday, C. (2006). Psychrophilic microorganisms: Challenges for life. *European Molecular Biology Organization* **7(4), 385-389.**

Ekici, Q. D., Paetzel, M. and Dalbey, R. E. (2008). Unconventional serine proteases: Variations on the catalytic Ser/His/Asp triad configuration. *Protein Science* **17, 2023-2037.**

Farah, Q., Kassab, E., Reiße, S., Atranikian, G. and Brueck, T. (2015). Characterization of a new, recombinant thermo-active subtilisin-like serine protease derived from *Shewanella arctica*. *Journal of Molecular Catalysis B: Enzymatic* **116, 16-23.**

Feller, G. (2003). Molecular adaptation to cold in psychrophilic enzymes. *Cellular and Molecular Life Sciences* **60, 648-662.**

Feller, G. (2010). Protein stability and enzyme activity at extreme biological temperatures. *Journal of Physics: Condensed Matter* **22, 1-17.**

Feller, G. (2013). Psychrophilic enzymes: From folding to function and biotechnology. *Scientifica* **13, 512840.**

Feller, G. and Gerday, C. (2003). Psychrophilic enzymes: Hot topics in cold adaptation. *Nature* **1, 200-208.**

Gupta, R., Beg, Q. K. and Lorenz, P. (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications. *Applied Microbiology Biotechnology* **59, 15-32.**

Hashim, N. H. F., Bharudin, I., Nguong, D. L. S., Higa, S., Abu Bakar, F. D., Nathan, S., Rabu., A., Kawahara, H., Illias, R. M., Najimudin, N., Mahadi, N. M. and Abdul Murad, A. M. (2013). Characterization of Afp1, an antifreeze protein from the psychrophilic yeast *Glaciozyma antarctica* PI12. *Extremophiles* **17(1), 63-73.**

Isaksen, G. V., Aqvist, J. and Brandsdal, B. O. (2014). Protein surface softness is the origin of enzyme cold-adaptation of trypsin. *Public Library of Science Computational Biology* **10(8), 1-9.**

Jaafar, N. R., Littler, D., Beddoe, T., Rossjohn, J., Md Illias, R., Mahadi, N. M., Mohamed Mackeen, M., Abdul Murad, A. M. and Abu Bakar, F. D. (2016). Crystal structure of fuculose aldolase from the Antarctic psychrophilic yeast *Glaciozyma antarctica* PI12. *Acta Crystallography* **72, 831-839.**

Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., Unni, K. N., Sajith, S., Priji, P., Josh, M. S. and Benjamin, S. (2013). Versatility of microbial proteases. *Advances in Enzyme Research* **1(3), 39-51.**

Kasana, R. C. (2010). Proteases from psychrotrophs: An overview. *Critical Reviews in Microbiology* **36(2), 134-145.**

Krissinel, E. and Henrick, K. (2004). Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallographic* **60, 2256-2268.**

Kumar, D., Savitri, N., Thakur, N., Verma, R. and Bhalla, T. C. (2008). Microbial proteases and application as laundry detergent additive. *Research Journal of Microbiology* **3(12), 661-672.**

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23, 2947-2948.**

Li, A. N. and Li, D. C. (2009). Cloning, expression and characterization of the serine protease gene from *Chaetomium thermophilum*. *Journal of Applied Microbiology* **106, 369-380.**

Lylloff, J. E., Hansen, L. B. S., Jepsen, M., Sanggaard, K. W., Vester, J. K., Enghild, J. J., Sørensen, S. J., Stougaard, P. and Glaring, M. A. (2016). Genomic and exoproteomic analyses of cold- and alkaline-adapted bacteria reveal an abundance of secreted subtilisin-like proteases. *Microbiology Biotechnology* **9(2), 245-256.**

Miao, L. L., Hou, Y. H., Fan, H. X., Qu, J., Qi, C., Liu, Y., Li, D. F. and Liu, Z. P. (2016). Molecular structural basis for the cold adaptedness of the psychrophilic β-glucosidase BglU in *Micrococcus antarcticus*. *Applied and Environmental Microbiology* **82(7), 2021-2030.**

Papaleo, E., Tiberti, M., Invernizzi, G., Pasi, M. and Ranzani, V. (2011). Molecular determinants of enzyme cold adaptation: Comparative structural and computational studies of cold- and warm-adapted enzymes. *Current Protein and Peptide Science* **12(7), 1-27.**

Petersen, T. N., Brunak, S., von Heijne, G. and Nielsen, H. (2011). SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nature Methods* **8, 785-786.**

Polgar, L. (2005). The catalytic triad of serine peptidases. *Cellular and Molecular Life Sciences* **62, 2161-2172.**

Pucci, F. and Rooman, M. (2017). Physical and molecular bases of protein thermal stability and cold adaptation. *Current Opinion in Structural Biology* **42, 117-128.**

Quevillon, E., Silventoinen, V., Pillai, S., Harte, N., Mulder, N., Apweiler, R. and Lopez, R. (2005). InterProScan: Protein domain identifier. *Nucleic Acids Research* **33, 116-120.**

Ran, L. Y., Su, H. N., Zhou, M. Y., Wang, L., Chen, X. L., Xie, B. B., Song, X. Y., Shi, M., Qin, Q. L., Pang, X., Zhou, B. C., Zhang, Y. Z. and Zhang, X. Y. (2014). Characterization of a novel subtilisin-like protease myroicolsin from deep sea bacterium *Myroides profundi* D25 and molecular insight into its collagenolytic mechanism. *The Journal of Biological Chemistry* **289, 6041-6053.**

Raschke, T. M. (2006). Water structure and interactions with protein surfaces. *Current Opinion in Structural Biology* **16, 152-159.**

Rawlings, N. D., Barrett, A. J. and Bateman, A. (2009). MEROPS: The peptidase database. *Nucleic Acids Research* **38, 227-233.**

Sabotic, J. and Kos, J. (2012). Microbial and fungal protease inhibitors-current and potential applications. *Applied Microbiology Biotechnology* **93, 1351-1375.**

Sarmiento, F., Peralta, R. and Blamey, J. M. (2015). Cold and hot extremozymes: Industrial relevance and current trends. *Frontiers in Bioengineering and Biotechnology* **3(148), 1-15.**

Siddiqui, K. S. (2015). Some like it hot, some like it cold: Temperature dependent biotechnological applications and improvements in extremophilic enzymes. *Biotechnology Advances* **33(8), 1912-1922.**

Siddiqui, K. S. and Cavicchioli, R. (2006). Cold-adapted enzymes. *Annual Revision of Biochemistry* **75, 403-433.**

Struvay, C. and Feller, G. (2012). Optimization to low temperature activity in psychrophilic enzymes. *International Journal of Molecular Sciences* **13, 11643-11665.**

Tina, K. G., Bhadra, R. and Srinivasan, N. (2007). PIC: Protein Interactions Calculator. *Nucleic Acids Research* **35, 473-476.**

Vojcic, L., Pitzler, C., Wirtz, G., Jakob, F., Martinez, R., Maurer, K. H. and Schwaneberg, U. (2015). Advances in protease engineering for laundry detergents. *New Biotechnology* **742, 1-6.**

Wang, W., Xia, M., Chen, J., Deng, F., Yuan, R., Zhang, X. and Shen, F. (2016). Data set for phylogenetic tree and RAMPAGE Ramachandran plot analysis of SODs in *Gossypium raimondii* and *G. arboretum*. *Data in Brief* **9, 345-348.**

Webb, B. and Sali, A. (2014). Comparative protein structure modeling using MODELLER. *In:* Current Protocols in Bioinformatics. John Wiley & Sons, Hoboken, New Jersey. **pp. 5.6.1-5.6.32.**

Willard, L., Ranjan, A., Zhang, H., Monzavi, H., Boyko, R. F., Sykes, B. D. and Wishart, D. S. (2003). VADAR: A web server for quantitative evaluation of protein structure quality. *Nucleic Acids Research* **31(13), 3316-3319.**

Yang, X. S., Chen, X. L., Xu, X. Z. and Zeng, R. Y. (2011). Cold-adaptive alkaline protease from the psychrophilic *Planomicrobium* sp. 547: Enzyme characterization and gene cloning. *Advances in Polar Science* **2011(22), 49-54.**

Yu, J., Zhou, Y., Tanaka, I. and Yao, M. (2010). Roll: A new algorithm for the detection of protein pockets and cavities with a rolling probe sphere. *Bioinformatics* **26(1), 46-52.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538