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Characterisation and molecular dynamic simulations of J15 asparaginase from *Photobacterium* sp. strain J15

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Genome mining revealed a 1011 nucleotide-long fragment encoding a type I L-asparaginase (J15 asparaginase) from the halo-tolerant *Photobacterium* sp. strain J15. The gene was overexpressed in pET-32b (+) vector in *E. coli* strain Rosetta-gami B (DE3) pLysS and purified using two-step chromatographic methods: Ni^{2+}-Sepharose affinity chromatography and Q-Sepharose anion exchange chromatography. The final specific activity and yield of the enzyme achieved from these steps were 20 U/mg and 49.2%, respectively. The functional dimeric form of J15-asparaginase was characterised with a molecular weight of ~70 kDa. The optimum temperature and pH were 25°C and pH 7.0, respectively. This protein was stable in the presence of 1 mM Ni^{2+} and Mg^{2+}, but it was inhibited by Mn^{2+}, Fe^{3+} and Zn^{2+} at the same concentration. J15 asparaginase actively hydrolysed its native substrate, L-asparagine, but had low activity towards L-glutamine. The melting temperature of J15 asparaginase was ~51°C, which was determined using denatured protein analysis of CD spectra. The K_m, K_{cat}, K_{cat}/K_m of J15 asparaginase were 0.76 mM, 3.2 s^{-1}, and 4.21 s^{-1} mM^{-1}, respectively. Conformational changes of the J15 asparaginase 3D structure at different temperatures (25°C, 45°C, and 65°C) were analysed using Molecular Dynamic simulations. From the analysis, residues Tyr_{24}, His_{22}, Gly_{23}, Val_{25} and Pro_{26} may be directly involved in the 'open' and 'closed' lid-loop conformation, facilitating the conversion of substrates during enzymatic reactions. The properties of J15 asparaginase, which can work at physiological pH and has low glutaminase activity, suggest that this could be a good candidate for reducing toxic effects during cancer treatment.

Key words: J15 asparaginase, *Photobacterium* sp., expression; purification, Molecular Dynamic (MD) simulations

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INTRODUCTION

L-asparaginase (EC 3.5.1.1) catalyses the hydrolysis of L-asparagine to L-aspartate and ammonia. The enzyme is widely used in the pharmaceutical and food industries. The discovery of asparaginase from guinea pig serum that can inhibit lymphoma, which is a type of blood cancer, in mice resulted in searches for L-asparaginase from different sources which might be useful for the treatment of human cancer (Broome, 1963). Although this enzyme can be found among organisms in different kingdoms, bacterial asparaginase has received more attention with regard to the treatment of cancer for several reasons: ease cultivation, economic feasibility, environmentally friendly and large scale production in a short period of time. L-asparaginase from different microbes have distinct characteristics based on adaptation to their habitat and ecological functions (Warangkar & Khobrade, 2010).

Bacterial asparaginase can be divided into type I and type II based on the cellular location and affinity towards different types of substrate (Campbell *et al.*, 1967). Type II periplasmic asparaginase displays high specific activity towards L-asparagine and is believed to have neoplastic activity; Hence, type II L-asparaginase is used for clinical applications. Meanwhile, cytoplasmic type I L-asparaginase is constitutively expressed and responsible for cell metabolism. The cytoplasmic type I L-asparaginase was less valuable since it has lower affinity toward the substrate L-asparagine (Schwartz *et al.*, 1966). The first crystal structure of type I bacterial L-asparaginase from the archaea *Pyrococcus horikoshii* was solved only at 2.6 Å resolution, while the structure of type II L-asparaginase has been studied extensively (Yao *et al.*, 2005). The enzyme was found to be dimeric rather than the common tetrameric architecture of type II L-asparaginase. Despite sharing a high degree of sequence similarity and overall folding, it is not clear why bacterial type II L-asparaginase functions as a tetramer whereas dimerisation provides all of the components of active sites in type I L-asparaginase (Michalska & Jaskolki, 2006).

The chemotherapeutic nature of asparaginase relies on the ability of the enzyme to maintain low levels of amino acids in the circulatory system, thus starving the cancer cell (Kidd, 1953). Cancers such as acute lymphoblastic leukaemia (ALL), Hodgkin's disease, acute myelocytic leukaemia, chronic lymphocytic leukaemia, acute myelomonocyticleukaemia, and lymphosarcoma, all of which lose the ability to synthesise amino acids themselves, depend heavily on amino acids from the outside in order to cope with their rapid growth (Stecher *et al.,* 1999; Duval *et al.*, 2002). The conversion ofthe amino acid L-asparagine to aspartic acid with L-asparaginase causes the apoptosis of cancer cells.

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Abbreviations: CD, circular dichroism; MD, molecular dynamics; RMSD, root mean square deviation

In spite of numerous reports on L-asparaginases obtained from Gram negative bacteria, the application of these enzymes is still limited due to the intrinsic glutaminase activity which partially contributes to some side effects during treatment. However, asparaginases from Gram negative bacteria such as *Escherichia coli* and *Erwinia carotovora* have been found to be the most effective as they possess lower toxicity and a strong preference to asparagine over glutamine (Duval *et al.*, 2002). Unfortunately, despite the wide usage of L-asparaginase in the past 30 years, the toxicity of the enzyme during treatment is still a major unresolved problem. Organ failure and hypersensitivity that can lead to death have been observed in patients during treatment. Although many improvements have been made to increase the pharmacokinetics, pharmacology and intracellular action of the enzyme, for example PEGylation (Veronese & Pasut, 2005) and encapsulation (Kwon *et al.*, 2009), none of these approaches eliminated the limitations associated with L-asparaginase during treatment. Therefore, scientists are struggling in the search for a more effective and less toxic L-asparaginase.

Halophilic bacteria possess many hydrolytic enzymes which are capable of functioning under conditions that lead to the precipitation and denaturation of most proteins. In addition, the saline nature of sea water is chemically closer to human blood plasma, and could provide a safer and more effective microbial enzyme with no or less toxicity when used for therapeutic applications. In the present study, we characterised the first reported L-asparaginase from the marine *Photobacterium* sp. strain J15 to identify its potential for therapeutic applications.

MATERIAL AND METHODS

Isolation and overexpression of gene. Isolation of full-length asparaginase gene was carried out using the primers Asn-F (5'-ATCGGGATCCATGGAAAGAAAACACATTTAC-3') and Asn-R(5'ATCGGAATTCTTTTAGTGAGTTAACTCACCC-3') with *Bam*HI and *Eco*RI restriction sites underlined, respectively. The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57.4°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The amplicons were digested with the restriction endonucleases *Bam*HI and *Eco*RI, and ligated into a pET-32b (+) vector that was digested with the same restriction endonucleases. The recombinant construct was then transformed into chemically competent *E. coli* TOP 10. Successful ligation was confirmed by double digestion and sequencing of the recombinant plasmid. The recombinant plasmid DNA was extracted from the transformants and then transformed into *E. coli* Rosetta-gami B (DE3) pLysS [genotype: F$^-$*ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1 ahpC* (DE3) *gor522*::Tn*10 trxB* pLysSRARE (Cam^R, Kan^R, Tet^R)] for protein expression.

J15 asparaginase assay. The asparaginase activity of J15 asparaginase was performed according to the standard protocol by Shirfin *et al.* (1974) with slight modifications. In this assay, the rate of ammonia released during the reaction was measured using Nessler reagent. A mixture of 0.05 ml of purified J15 asparaginase, 0.5 ml 50 mM phosphate buffer (pH 7.4), 0.45 ml deionised water (ddH_2O) and 0.05 ml of 0.189 M L-asparagine was incubated for 30 min at 37°C. The reaction was stopped by the addition of 0.05 ml of 1.5 M trichloroacetic acids. After centrifugation at 15 294 × *g*, 0.1 ml of the supernatant was diluted to 2.15 ml with ddH_2O and treated with 0.25 ml of Nessler's reagent. The colour reaction was allowed to develop for 1 min and the absorbance was read at 430 nm with a UV visible spectrophotometer (Ultrospec 2100, GE Healthcare, and USA). The standard was prepared by plotting the absorbance at 436 nm of the standard versus ammonia (NH_3) concentration. Ammonia liberated in the sample was extrapolated from a standard curve. One unit (U) of L-asparaginase activity was defined as the rate of liberating 1 μmol of ammonia per min under the standard assayed conditions.

Purification of J15 asparaginase. The initial purification step was performed using affinity chromatography with 10 ml of Ni^{2+}-Sepharose HP (GE Healthcare, USA). The cell pellet from a 200 ml culture was resuspended in 20 ml of binding buffer (50 mM phosphate buffer, 0.5 M NaCl, 70 mM imidazole, pH 7.4). The crude enzyme was loaded into 10 ml of Ni^{2+}-Sepharose HP resin packed into a XK 16/20 column which was previously equilibrated with 50 ml [(equal to 5 column volume (CV)] of binding buffer at a flow rate of 1 ml/min. The unbound protein was washed with 10 CV of binding buffer. The bound protein was eluted under a gradient step using elution buffer (50 mM phosphate buffer, 0.5 M NaCl and 500 mM imidazole, pH 7.4).

The eluted fractions were treated with thrombin (1 U/mg of fusion protein) to remove the Trx-tag at the N-terminal of J15 asparaginase at 20°C for 20 h and dialysed against phosphate buffer (20 mM phosphate buffer, 50 mM NaCl, pH 6.5). The thrombin-digested fusion protein was loaded into a 5 ml Q-Sepharose column (GE Healthcare, USA) which was pre-equilibrated with binding buffer (20 mM phosphate buffer, 50 mM NaCl, pH 6.5). Non-adsorbed protein was washed off with equilibration buffer followed by elution of the bound protein using elution buffer (20 mM phosphate buffer, 1 M NaCl, pH 6.5). Collected fractions (2 ml) were assayed for asparaginase activity. Protein subunits and molecular mass of the purified protein was determined by denaturing gel electrophoresis (12% SDS/PAGE) and MALDI-TOF/TOF mass spectrometry, respectively. Protein oligomerisation was analysed using native gel electrophoresis (10% Native-PAGE). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Characterisation of J15 asparaginase. The purified J15 asparaginase in 50 mM phosphate buffer (pH 7.4) was assayed at various temperatures ranging from 15°C to 55°C using 5°C intervals for 30 min, with L-asparagine as a substrate. The stability of the J15 asparaginase was determined by pre-incubating purified J15 asparaginase for up to 7 h at 20°C, 25°C and 30°C in 50 mM phosphate buffer (pH 7.4) prior to the asparaginase assay. The pH profile was determined by assaying the purified J15 asparaginase using 50 mM of various buffer systems: acetate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–8.0), Tris/HCl buffer (pH 8.0–9.0) and glycine–NaOH buffer (pH 9.0–12.0). The effects of various metal ions on J15 asparaginase activity were tested by treating purified J15 asparaginase with 1 mM of metal ions for 30 min at 25°C, pH 7.0. The residual activity was measured using the L-asparaginase assay. The purified J15 asparaginase was treated with different types of inhibitors (PMSF, EDTA, β-mercaptoethanol and SDS) at a final concentration of 1 mM for 30 min at 25°C. The residual activity was measured colorimetrically. The purified J15 asparaginase was also subjected to different types of substrates to check the substrate specificity at 25°C.

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J15             --------------------MERKHIYIAYTGGTIG----MQKSDHGYVPVAGFMQKQLE 36
VC              --------------------MARKHIYIAYTGGTIG----MKKSDHGYVPVAGFMEKQLA 36
EC              MGSSHHHHHHSSGLVPRGSHMQKKSIYVAYTGGTIG----MQRSEQGYIPVSGHLQRQLA 56
PH              -----------------------MRILILGMGGTIA----SVKGERGYESALS-VSKILK 32
ER              --------------------ADKLPNIVILATGGTIAGSAATGTQTTGYKAGALGVDTLIN 41
HP              -------------------MAQNLPTIALLATGGTIAGSGVDASLGS-YKSGELGVKELLK 41
                                       * :   ****.           * .    :.  :

J15             SMPEFQRPEMPKFTIHEYEPLIDSSDMSPADWQRIADDIKENY--DEYDGFVILHGTDTM 94
VC              SMPEFHRPEMPLFTIHEYDPLMDSSDMTPADWQLIADDIAANY--DKYDGFVILHGTDTM 94
EC              LMPEFHRPEMPDFTIHEYTPLMDSSDMTPEDWQHIAEDIKAHY--DDYDGFVILHGTDTM 114
PH              LAGISSEAKIEARDLMN----VDSTLIQPSDWERLAKEIEKEV--WEYDGIVITHGTDTM 86
ER              AVPEVKKLANVKGEQFSN---MASENMTGDVVLKLSQRVNELLARDDVDGVVITHGTDTV 98
HP              AIPSLNKIARIQGEQVSN---IGSQDMNEEIWFKLAQRAQELLDDSRIQGVVITHGTDTL 98
                      .          .    : *  :       ::.           :*.** *****:

J15             AYTASALSFMFENLDKPVIVTGSQIPLAELRSDGQSNLLNSLHIAAN--YPINEVTVFFN 152
VC              AYTASALSFMFENLGKPVIVTGSQIPLADLRSDGQANLLNALHVAAN--YPINEVTLFFN 152
EC              AYTASALSFMLENLGKPVIVTGSQIPLAELRSDGQINLLNALYVAAN--YPINEVTLFFN 172
PH              AYSASMLSFMLRNPPIPIVLTGSMLPITEKNSDAPFNLRTALEFVK---LGIRGIYIAFN 143
ER              EESAYFLHLTVKS-DKPVVFVAAMRPATAISADGPMNLLEAVRVAGDKQSRGRGVMVVIN 157
HP              EESAYFLNLVLHS-TKPVVLVGAMRNASSLSADGALNLYEAVSVAVNEKSANKGVLVVMD 157
                  :*  * : ...   *::...:    :   :*.  **  :: ..       . : : ::

J15             NQLIRGNRSTKAHADGFGAFISPNMPPLLEAGINIQLN-NVELDK--KPEGKFKVHNITP 209
VC              NRLMRGNRSRKSHADGFSAFSSPNLPPLLEAGINIELSTNVKVDE--KPSGEFKVNPITP 210
EC              NRLYRGNRTAKAHADGFDAFASPNLPPLLEAGIHIRRL-NTPPAP--HGEGELIVHPITP 229
PH              GKVMLGVRASKIRSMGFDAFESINYP--NVAEIKDDKLRILHIPD--FYGDEFFSDIKYE 199
ER              DRIGSARYITKTNASTLDTFRANEEGYLG-VIIGNRIYYQNRIDKLHTTRSVFDVRGLTS 216
HP              DTIFSVREVVKTHTTHVSTFKALNSGAIGSVYYGKTRYYMQPLRK-HTTESEFSLSQLKT 216
                . :       * .:  ..:* : :      .                    . :

J15             --QPIGVITMYPGISPEVIRNTLRQPVNAMILLTFGVGNAPQNPELLAQLKEAADRGVIV 267
VC              --QPIGVITMYPGISHEVIRNTLLQPVNAMILLTFGVGNAPQNPELLAQLKAASERGVIV 268
EC              --QPIGVVTIYPGISADVVRNFLRQPVKALILRSYGVGNAPQNKAFLQELQEASDRGIVV 287
PH              --PKVLVIKLIPGLSGDIVREALRLGYKGIILEGYGVGGIPYRGTDLFEVVSSISKRIPV 257
ER              -LPKVDILYGYQDDPEYLYDAAIQHGVKGIVYAGMGAGSVSVR--GIAGMRKALEKGVVV 273
HP              PLPKVDIIYTHAGMTPDLFQASLNSHAKGVVIAGVGNGNVSAG--FLKAMQEASQMGVVI 274
                    : ::    . .  :    :    :.::    * *. .     :  :  : .  : :

J15             MNLTQCLSGKVNMGGYATGCALADAGVLSGYDMTPEAALAKLHFLLSQDLPLETIRSLMQ 327
VC              VNLTQCLAGKVNMGGYATGCALADAGVISGYDMTPEAALAKLHYLLSQNLSYEEVKAKMQ 328
EC              VNLTQCMSGKVNMGGYATGNALAHAGVIGGADMTVEATLTKLHYLLSQELDTETIRKAMS 347
PH              VLTTQAIYDGVDLQRYKVGRIALEAGVIPAGDMTKEATITKLMWILGHTKNIEEVKQLMG 317
ER              MRSTRTGNGIVPP-------DEELPGLVS-DSLNPAHARILLMLALTRTSDPKVIQEYFH 325
HP              VRSSRVGSGGVTSG----EIDDKAYGFITSDNLNPQKARVLLQLALTKTNDKAKIQEMFE 330
                :  ::   . *             *.:   .:.   :   *   * :      ::  :

J15             QNLRGELTH-- 336
VC              QVLRGEMTL-- 337
EC              QNLRGELTPDD 358
PH              KNITGELTRVS 328
ER              TY--------- 327
HP              EY--------- 332
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Figure 1. Multiple sequence alignment of selected type I L-asparaginases from different species.
The alignment was generated using CLUSTALW (Thompson *et al.,* 1994). Fully conserved region were indicated by (*), deleted region by (–) and conservation of strong and weak groups is denoted by (:) and (.) respectively. The sequences of five intracellular L-asparaginases were shown; J15: *Photobacterium* sp. strain J15; VC, *Vibrio cholerae*; EC, cytoplasmic *E. coli*; PH, *Pyrococcus horikoshii*; ER, *Erwinia crysanthemi*; HP, *Helicobacter pylori*.

Kinetic analysis of J15 asparaginase. The purified J15 asparaginase in 20 mM phosphate buffer (pH 7.0) was incubated with different concentrations of L-asparagine (2 mM, 4 mM, 6 mM, 8 mM and 10 mM) at 25°C using various incubation times (10, 20, 30, 40, 50 and 60 min). The amount of ammonia released was detected by the standard L-asparaginase assay. The kinetic parameters K_m and K_{cat} were calculated using non-linear regression analysis of experimental steady state data. The linear regression analysis was achieved using a Lineweaver-Burke plot ($1/V_o$ against $1/[S]$).

***T*$_m$ and secondary structure content of J15 asparaginase analysis by circular dichroism (CD).** Purified J15 asparaginase in 10 mM sodium phosphate buffer (pH 7.0) was analysed with the J-810 spectropolarimeter (Jasco, Japan) for CD spectral analysis. The warm-up periods of 10°C to 90°C and a wavelength scan of 180 to 250 nm were applied. The variable temperature measure-

ment of J15 asparaginase was performed by employing 10 mm cells after checking the CD value at 220 nm. The warm-up period was 10°C to 90°C, with 1 degree incrementsper minute; the wavelength was set to 220 nm. The concentration was 0.2 mg/ml and the top of the cell was completely closed using a cap. Datapitch, bandwidth, response, scanning speed, and accumulation were set as 0.1°C, 1 nm, 8 S, 1°C/min, 8 times, respectively. Far-UV CD spectra were recorded at a wavelength within the region from 190-240 nm, with a 1.0 mm path length cuvette containing the native enzyme solutions. The baseline reading was corrected with the respective blank. The raw data from CD spectra were analysed against the reference database from http://perry.freeshell.org.

Homology modelling and MD simulation study. Comparative modelling, molecular docking and Molecular Dynamic (MD) simulations were conducted using the YASARA program (YASARA Biosciences, Austria). The template of *Vibrio cholerae* L-asparaginase (2OCD) was retrieved from PDB (http://www.rcsb.org/), while ligands (L-asparagine) were taken from the Pubchem compound (http://www.ncbi.nlm.nih.gov/pccompound). Local docking was performed using Auto-Dock 4.2 (Morris *et al.,* 1998) with the default docking parameter and point charges assigned according to the AMBER03 force field (Duan *et al.,* 2003). The setup was done using the YASARA molecular modelling program (Krieger *et al.*, 2002). MD simulation was carried out for 20 ns at different temperatures(25°C, 45°C, and 65°C) in an aqueous environment. The simulation parameters such as water density and counter ion concentrations (Na^+ and Cl^-) were 0.997 g/L and 0.9%, respectively. The simulation was run in truncated orthorhombic cell under periodic boundary conditions. AMBER03 N force field was applied to all atoms in the system with 7.86 Å cut-offs andnon-bonded interactions. The pKa values for Asp, Glu, Hisand Lys residues were predicted and the protonation states were assigned according to pH 7.0, which is the optimum pH of J15 asparaginase.

RESULTS AND DISCUSSION

Expression and purification of J15 asparaginase

The genome of *Photobacterium* sp. strain J15 was partially sequenced and a 1011 bp open reading frame (ORF) fortype I L-asparaginase was revealed. This ORF encodes a predicted protein of 336 amino acids with 82% identity to L-asparaginase from *Vibrio cholerae*. Figure 1 shows the multiple sequence alignment of the available L-asparaginase from different bacterial species. The conserved residues which are likely to be involved in the catalysis of substratesare Thr_{14}, Tyr_{24}, Thr_{91}, Asp_{92} and Ser_{117}; Lys_{163} was found on the J15 asparaginase sequence in agreement with the *E. coli* asparaginase structure (Sanches *et al.*, 2007). The other residues involved in catalysis are residues 6–38, which contain a flexible loop.

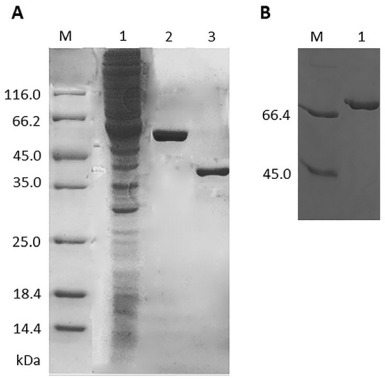

Figure 2. Polyacrylamide gel electrophoresis of J15 asparaginase.
(**a**) SDS/PAGE (12%) of J15 asparaginase. M, Unstained protein molecular marker; Lane 1, crude extract; Lane 2, Purified J15 asparaginase after Nickel Sepharose chromatography 3, purified J15 asparaginase after anion exchange chromatography; (**b**) Non-denatured SDS/PAGE (10%) of J15 asparaginase. M, Bovine serum albumin and protease from *Bacillus* sp.; Lane 1, Purified protein from Q-Sepharose anion exchange chromatography. The size of the protein was estimated as ~35 kDa and ~70 kDa when analyzed with SDS/PAGE and Non-denatured SDS/PAGE, respectively.

This loop functions like a lid, as it opens and closes in a ligand-dependent manner (Offman *et al.*, 2010). The purification of the enzyme gave a final yield and fold of 49.2% and 10, respectively. The purified J15 asparaginase after affinity chromatography and ion exchange chromatography was electrophoresed as a single band with an estimated size of about 35 kDa on 12% SDS-PAGE. The J15 asparaginase was in dimeric form with molecular mass of about 70 kDa (Fig. 2). The purity of the J15 asparaginase preparation was assessed by MALDI-TOF/TOF analysis where the hits from the Ludwig NR database forthe fragment HIYIAYTGGTIGMQK gave the ion score of 44.4 and showed 65% similarity with L-asparaginase from *Vibrio cholerae* (data not shown).

Biochemical properties of J15 asparaginase

Effect of temperature, pH, substrate specificity and metal ions

The purified J15 asparaginase was active at temperatures between 20°C to 50°C, with an optimal temperature of 25°C (Fig. 3a). The activity of J15 asparaginase gradually decreased with increasing temperature. This may because of the structural changes of the protein which led to altered folding and consequently rendered the protein inactive. The purified J15 asparaginase was also incubated at near optimum temperatures of 20°C, 25°C and 30°C to determine the extent of temperature resistance of J15 asparaginase. The enzyme was stable for 3 h at 20°C. At 25°C and 30°C, J15 asparaginase was stable for 1h only. The half-life of the enzyme was 4 h, 3.5 h and 3 h at 20°C, 25°C and 30°C, respectively (Fig. 3b). The enzyme was active at a broad pH range (pH 6.0 to pH 8.0) and had optimum activity at physiological pH (pH 7.0) (Fig. 4). The ability of an enzyme to work best at physiological pH is more favourable since physiological conditions comprise one of the requirements for anti-tumour activity. Most L-asparaginases that

Table 1. Purification table of the intracellular expressed fusion J15 asparaginase from *Photobacterium* sp. strain J15.

Steps	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude extract	12	776.5	349.2	2.2	1.0	100.0
Ni^{2+}-Sepharose	8	599.0	56.0	10.7	4.9	77.1
Q-Sepharose	5	382.5	18.5	20.0	10.0	49.2

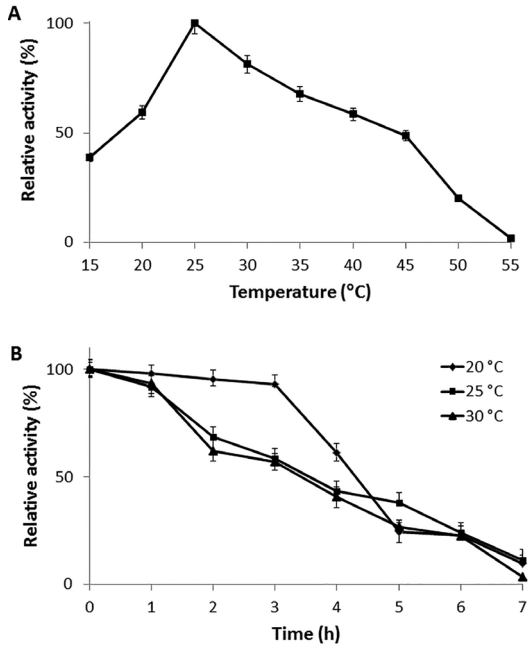

Figure 3. The temperature profile of purified J15 asparaginase.
(**a**) The activity of the enzyme at different temperature. The purified J15 asparaginase was assayed at various temperature (15–55°C) using L-asparagine as substrate. The relative activity is expressed as the percentage of the enzyme activity at the optimum temperature (25°C). (**b**) Thermal stability of J15 asparaginase at 20°C (◆), 25°C (■) and 30°C (▲) at different incubation time.

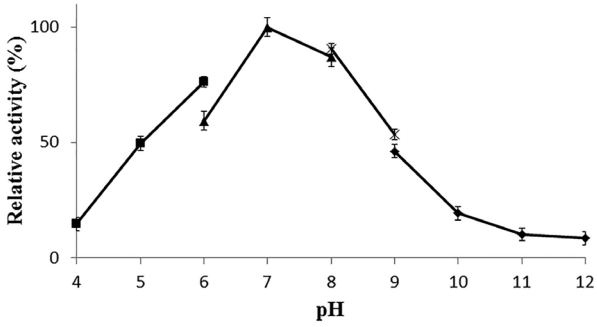

Figure 4. Effect of pH on J15 asparaginase activity.
The buffers used were sodium acetate buffer, (■); pottassium phosphate buffer (▲); Tris-HCl buffer (×) and glycine-NaOH buffer (◆).

have been characterised, such as those from *Erwinia* sp. and *E. coli,* exhibit optimal pH at alkaline conditions (pH 8.0 to pH 9.0) and acidic conditions (pH 5.0 to pH 6.0) (Muller and Boos, 1998). Moreover, L-asparaginase from *Bacillus licheniformis (*Mahajan *et al.,* 2012), *Helicobacter pylori* (Cappelletti *et al.,* 2008) and *Staphylococcus* sp. (Prakasham *et al*., 2007) share the same optimal pH as J15 asparaginase. J15 asparaginase exhibits the highest activity toward its natural substrate (L-asparagine), while L-glutamine is weakly catalysed (less than 5%). This property makes J15 asparaginase potentially very useful in medical applications.The addition of univalent and divalent metal ions did induce enhanced J15 asparaginase activity (Table 2). However, Mn^{2+}, Zn^{2+} and Fe^{2+} were very detrimental to enzymatic activity. The inhibition of J15 asparaginase with different metal ions, and the lack of effect of the metal chelator EDTA, indicated that J15 asparaginase is highly unlikely to be a metalloenzyme.

Table 2. The effect of substrates, metal ions, and inhibitors on J15 asparaginase activity.
The result is the average of three independent expriment with less than 5% standard deviation.

Treatment	Relative activity (%)
L-asparagine	100.0
L-glutamine	4.9 ± 1.98
L-glutamic acid	0.0
L-aspartic acid	0.0
EDTA	97.9 ± 1.52
PMSF	99.3 ± 2.93
β-mercaptoethanol	72.3 ± 1.20
SDS	9.2 ± 0.52
Li^{+}	71.8 ± 0.73
Na^{+}	78.4 ± 2.65
K^{+}	51.2 ± 0.91
Rb^{+}	62.7 ± 1.2
Cs^{+}	59.9 ± 1.56
Mg^{2+}	97.4 ± 1.97
Ca^{2+}	50.7 ± 0.31
Sr^{2+}	59.4 ± 0.87
Mn^{2+}	17.8 ± 1.54
Co^{2+}	79.0 ± 0.91
Ni^{2+}	96.8 ± 0.57
Cu^{2+}	64.6 ± 3.10
Zn^{2+}	16.1 ± 2.45
Fe^{3+}	0.0

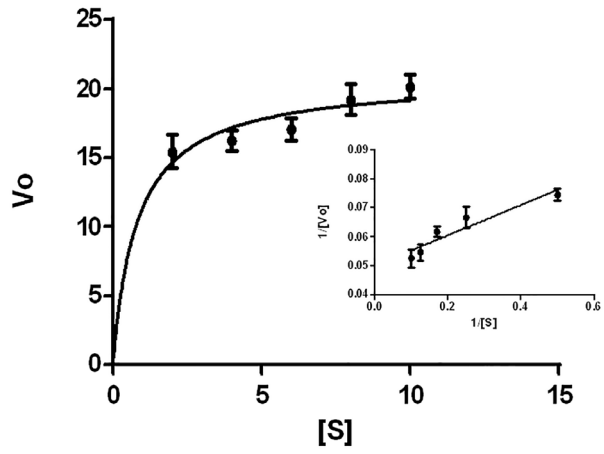

Figure 5. Plot of the reaction velocities (V_o) *versus* substrate concentration [S] fitted to the Michaelis–Menten equation and determination of K_m and V_{max} of purified J15 asparaginase by nonlinear regression analysis.
(Inset) The corresponding Lineweaver–Burk plot for J15 asparaginase catalyzed reaction.

Kinetic analysis

The effectiveness of a particular L-asparaginase against tumour cells can be predicted from the *in vitro* affinity towards the substrate L-asparagine. The K_m, K_{cat} and K_{cat}/K_m of L-asparaginase were found to be 0.76 mM, 3.2 s^{-1} and 4.21 $s^{-1}mM^{-1}$, respectively (Fig. 5). The reported K_m values for *Erwinia aroideae* NRRL Q-138 (Peterson & Ciegler, 1969), *Vibrio succinogen* (Willis & Woolfolk, 1974)

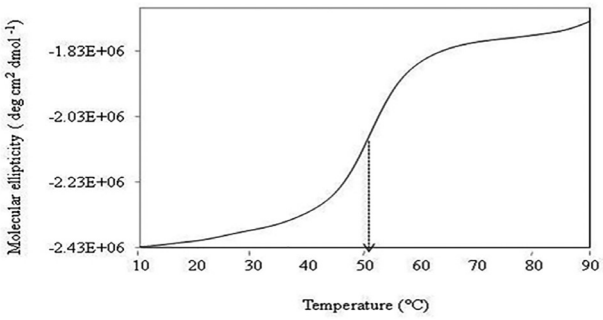

Figure 6. The CD spectra of J15 asparaginase as a function of temperature at 220 nm.
Denatured protein analysis of J15 asparaginase. The dash arrow indicates the melting point (T_m) of J15 asparaginase.

and *Erwinia crysanthemi* 3937 (Kotzia & Labrou, 2007) were 0.03 mM, 0.074 mM and 0.058 mM, respectively, which were lower and more effective than that for J15 asparaginase. Meanwhile, higher K_m values of 2.5 mM and 3.5 mM for L-asparaginase from *Corynebacterium glutamicum* and *E. coli,* respectively, have been reported (Willis & Woolfolk, 1974). Thus, purified J15 asparaginase has comparable efficiency in degrading asparagine with *E. coli* asparaginase, which is clinically used in cancer treatment regimens.

Secondary structure and T_m prediction by circular dichroism

CD spectra are widely used to determine the equilibrium between helical structures and unordered conformations (Schröder*et al.,* 2001). The CD spectrum (molecular ellipticity) of J15 asparaginase was analysed as a function of temperature at 220 nm. The wavelength 220 nm was selected to monitor the transition of the α-helical structure to unordered structures as they exhibited characteristic signals at this wavelength. The sigmoidal shape of the resulting denaturation curve indicated a monophasic helix coil transition of J15 asparaginase within the analysed temperature range. The fitting lines were plotted from the stable region to the range of denaturation (30°C to 55.8°C) in order to obtain a melting temperature (T_m) value. The T_m value for J15 asparaginase was 50.94°C (Fig. 6). According to the second law of thermodynamics, the free energy change (ΔG) at a constant temperature and pressure is $\Delta G = \Delta H - T\Delta S$. When a protein starts to unfold due to heating, the process will go through an intermediate state; thus, the free energy change (ΔG) at equilibrium was zero. As a result, J15 asparaginase showed a melting temperature of 50.94°C, as the unfolding enthalpy (ΔH) and entropy (ΔS) were 58.67 kcal/mol and –0.18 kcal/mol/K, respectively. One of the most successful applications of CD in characterising a protein depends upon the remarkable sensitivity of the far-UV to the backbone conformation of proteins to reflect the secondary content of the protein (Sreerama *et al.,* 2000). The CD signal at certain wavelength (193.0 nm, 196.0 nm, 207.0 nm, 211.0 nm and 234.0 nm) was required to estimate secondary structure content in J15 asparaginase against the reference database (http://perry.freeshell.org). The purified J15 asparaginase comprised of 21.3% α-helix, 27.1% β-sheet, 12.5% turn and 33.3% random (Raussens *et al.*, 2003).

Molecular dynamic simulation of J15 asparaginase as a function of temperature

Structural alignment using YASARA software demonstrated that the RMSD of corresponding Cα atoms between J15 asparaginase and L-asparaginase from *Vibrio cholerae* (Pdb: 2OCD) was 0.431 for 301 Cα atoms. The low RMSD value indicated a significant structural similarity as the sequence identity over the fit region was 73%. MD simulations of J15 asparaginase for 20 ns were carried out at three different temperatures (25°C, 45°C and 65°C) to determine the structural behaviour in an aqueous environment at the respective temperatures. The conformational changes of J15 asparaginase at different temperature were analysed based on the RMSD value, the radius of gyration, the solvent accessible surface and the calculated B-factor. J15 asparaginase was most stable at 25°C, which is the optimum temperature for hydrolysis of the substrate. This was proven by the small fluctuations of the RMSD value and increased withtemperature. There was a significant difference when comparing the root mean square deviation (RMSD) of the Cα backbone for the simulation run at different temperatures (Fig. 7a). Throughout these experiments, the RMSD value ranged from 1.2 Å to 4.0 Å, indicating that molecules in J15 asparaginase undergo conformational alterations in the bonding and dihedral angles of its backbone. The changes insize and compactness of J15 asparaginase were examined by its radius of gyration and solvent accessible surface (Fig. 7b and 7c).

At temperatures above 25°C, J15 asparaginase started to loss its notable compactness (2Å increment) and more residues were exposed to the environment; The reason for this is that above this temperature, J15 asparaginase starts to denature and many bonds begin to break apart because of the excessive amount of heat in the system. The root mean square of fluctuation per residue was calculated in terms of B-factor, and also showed a similar trend (Fig. 7d). Residues 127–141 and 301–314, which correspond to α4 helix and α7 helix, showed significant fluctuations: 127–641 $Å^2$ and 180–347 $Å^2$, respectively. At 25°C, J15 asparaginase retained its structure and was less flexible, especially at residues 6–38, which are important for catalysis. However, as the temperature increased, the bonds that stabilise the 3D structure of J15 asparaginase were disrupted and the structure became less rigid; as a result, the active site was distorted and it was unlikely that hydrolysis would occur. MD simulation was also carried out at 25°C for 20 ns to analyse the behaviour of the flexible lid-loop at positions 6–38, and also in comparison with *E. coli* L-asparaginases that were reported by Aung and coworkers (2000) and Offman and coworkers (2010). The mobile loop at the active site in *E. coli* asparaginase adopts an open conformation in the absence of substrate and a closed formation upon addition of the substrate. This indicates that the loop opens and closes in a ligand-dependent fashion. One of the key residues identified was Tyr_{25} in *E. coli* L-asparaginase and Tyr_{24} in J15 asparaginase. Calculating the B-factor per residue from the MD simulation allowed analysis binding of the lid-loop in J15 asparaginase with substrate and in J15 asparaginase without substrate (control). The data indicate that the mobile loop region displays less flexibility to direct the orientation of a substrate to initiate hydrolysis upon binding of a ligand. This was indicated by lower fluctuation of the calculated B-factor per residue at the region of 6–38 (Fig. 8). The stable, closed conformation of a lid-loop was observed when a substrate molecule is present in the active site throughout the sim-

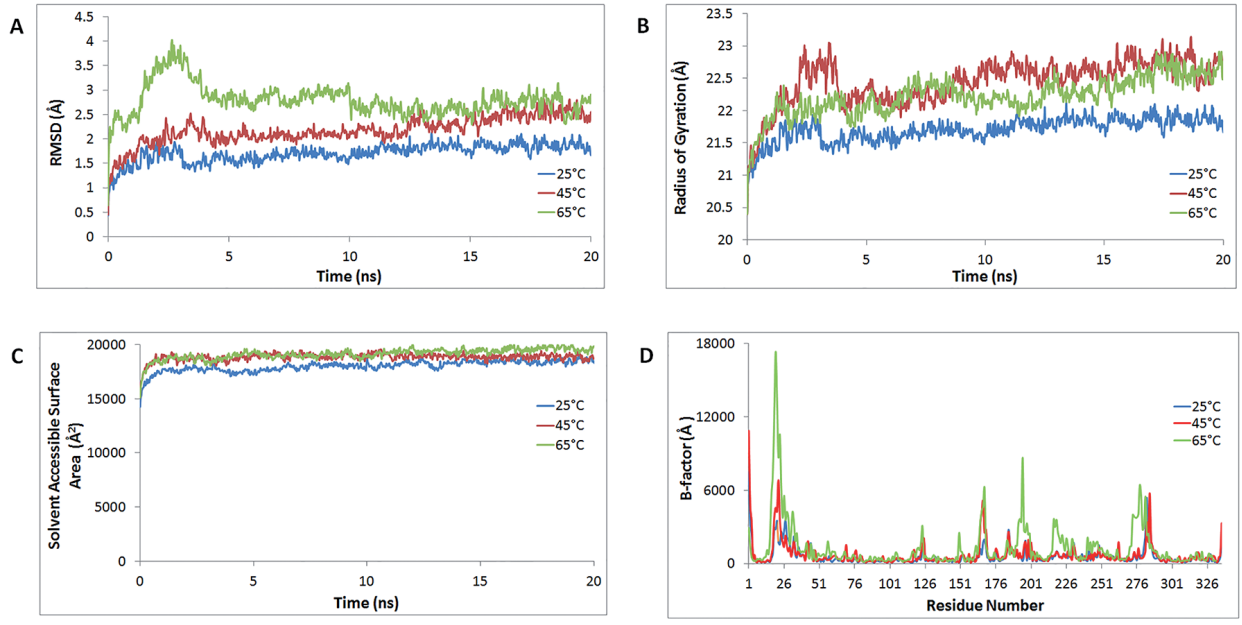

Figure 7. Conformational changes of J15 asparaginase at different temperature (25°C, 35°C and 65°C).
(**a**) RMSD of Cα-backbone atoms of J15 asparaginase as a function of time (20 ns); (**b**) Radius of gyration as a function of time (20 ns); (**c**) Solvent accessible surface area on function of time (20 ns); (**d**) B-factors of J15 asparaginase as a function of time (20 ns).

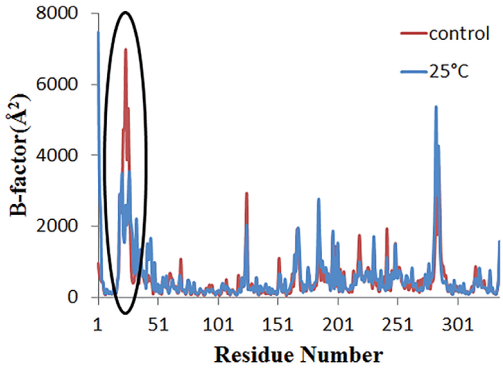

Figure 8. The conformational changes of flexible loop with and without a ligand (L-asparagine).
Calculated B-factors of J15 asparaginase residues during simulation of 20 ns. Molecular movement (Å²) of residues at lid loop region in J15 asparaginase structure (in circle).

ulation times. In addition, based on the significant reduction of fluctuation in the B-factor residue, other residues may also be involved in regulating the orientation of the substrate. Besides Tyr_{24} (6983.6–2603.1 Å²), the other residues involved are His_{22} (4741–1749 Å²), Gly_{23} (4728–1562 Å²), Val_{25} (3909–2040 Å²) and Pro_{26} (5325–2679 Å²). These residues, which are located at the entrance of the tunnel, are potential candidates for studying the impact of the lid-loop and the effect of their mutation on J15 asparaginase activity.

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