Journal of Genetic Engineering and Biotechnology (2017) **15**, 231–237

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and
National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ORIGINAL ARTICLE

System analysis of salt and osmotic stress induced proteins in *Nostoc muscorum* and *Bradyrhizobium japonicum*

Vipin Kaithwas [a], **Krati Bhardwaj** [a], **Durgesh Gupta** [b], **Santosh Bhargava** [a,*]

[a] *Division of Microbiology, Department of Botany, Government Motilal Science College, Bhopal, MP 462008, India*
[b] *Bioinformatics Centre, Barkatullah University, Bhopal, MP 462026, India*

Received 19 August 2016; revised 26 October 2016; accepted 19 December 2016
Available online 2 February 2017

KEYWORDS

Amino acids;
Salt induced proteins;
Sucrose induced proteins

Abstract In this study the proteome response of the two diazotrophic organism's *viz*. *Nostoc muscorum* and *Bradyrhizobium japonicum* exposed to salt (NaCl) and osmotic (sucrose) stresses was compared. Out of the total over expressed proteins; we have selected only three over expressed proteins *viz*. GroEL chaperonin, nitrogenase Mo-Fe protein and argininosuccinate synthase for further analysis, and then we analyzed the amino acid frequencies of all the three over expressed proteins. That led to the conclusion that amino acids e.g. alanine, glycine and valine that were energetically cheaper to produce were showing higher frequencies. This study would help in tracing the phylogenetic relationship between protein families.

1. Introduction

Protein molecules are essential for maintenance of growth, reproduction, synthesis of other essential biomolecules and to prevent oxidative damage [1]. Any up shift or down shift in the environmental factor resulted in protein damage, which ultimately disturbs the biological functions or protein functions [2–4]. Various living organisms have inbuilt capacity to overcome various environmental stresses by synthesizing molecular chaperones, which play a major role in the cellular protein homeostasis. These molecular chaperones recognized non-native proteins and binds with them to retain proper conformation of the native proteins thereby providing protection to the organism to cope with changing environment [5].

Stress response on the one hand leads to protein damage and down regulation of certain genes [6], on the other hand it activates the expression of certain genes commonly known as stress genes [7]. As a consequence of activation of the stress genes; stress proteins are synthesized to overcome stress factor [8]. Owing to some historical reasons stress proteins are normally classified as heat shock proteins or heat stress proteins (HSPs). These HSPs are further classified into different families, according to their molecular masses. The main families are HSP100, HSP90, HSP70 (chaperones), HSP60 (chaperonins), HSP40 (DnaJ) and some small HSPs. HSP is present in all the three domains of life and known to play a critical role in cell protection against various environmental stresses as well as in maintaining cellular homeostasis [9,10].

* Corresponding author. Fax: +91 755 2551460.
E-mail address: santoshbhargava@hotmail.com (S. Bhargava).
Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2016.12.007

Stress proteins normally exist in low concentrations within the cell; the concentration of stress proteins enhanced many folds under stress conditions. Thus, it is suggested that environmental stress activates some specific genes that are necessary for synthesis and over accumulation of stress proteins [11–13].

Stress response is usually associated with the protein modification and DNA processing. The mechanism of stress response is almost similar in all the three domains of life. This indicates that it comprises an evolutionary highly conserved mechanism that provides protection to the cell from unfavorable environmental conditions. The proteome response of cell under stress conditions involves induction, post translation modification, protein–protein interaction and protein-DNA interaction [14].

Since the dawn of evolution various organisms has been exposed to changing environmental factors. These environmental stresses lead to the origin and evolution of highly conserved stress proteins. Thus, it seems that a core stress proteome appeared early in cellular evolution, supporting life under stressful environment. The origin and evolution of other stress proteins may involve mutation or adaptive radiation of genes. In spite of their common ancestry, some stress proteins in contemporary species are less well conserved, the reason being the origin of the different cellular structure is followed by metabolic plasticity and signal transduction mechanism.

In this study salinity and osmotic stress responses at the proteome level and amino acids frequencies were analyzed in two diazotrophic strains *viz*. *Bradyrhizobium japonicum* and *Nostoc muscorum*. It is known that both the stresses caused deleterious effect on protein conformation. Therefore, we have selected these stresses to study proteome changes and their evolutionary history.

2. Materials and methods

2.1. Organisms and growth conditions

The cyanobacterium *N. muscorum* used in the present study is a gift from Prof. Surendra Singh, Department of Biological Sciences, Rani Durgawati University, Jabalpur (M.P.), India. The culture of *B. japonicum* was purchased from culture collection of National Chemical Laboratory, Pune, India. The cyanobacterium *N. muscorum* is grown and maintained in Chu No. 10 medium [15] and the culture of *B. japonicum* on AG growth medium [16] at 28 °C. The ionic stress in the growth medium is created with the addition of NaCl and

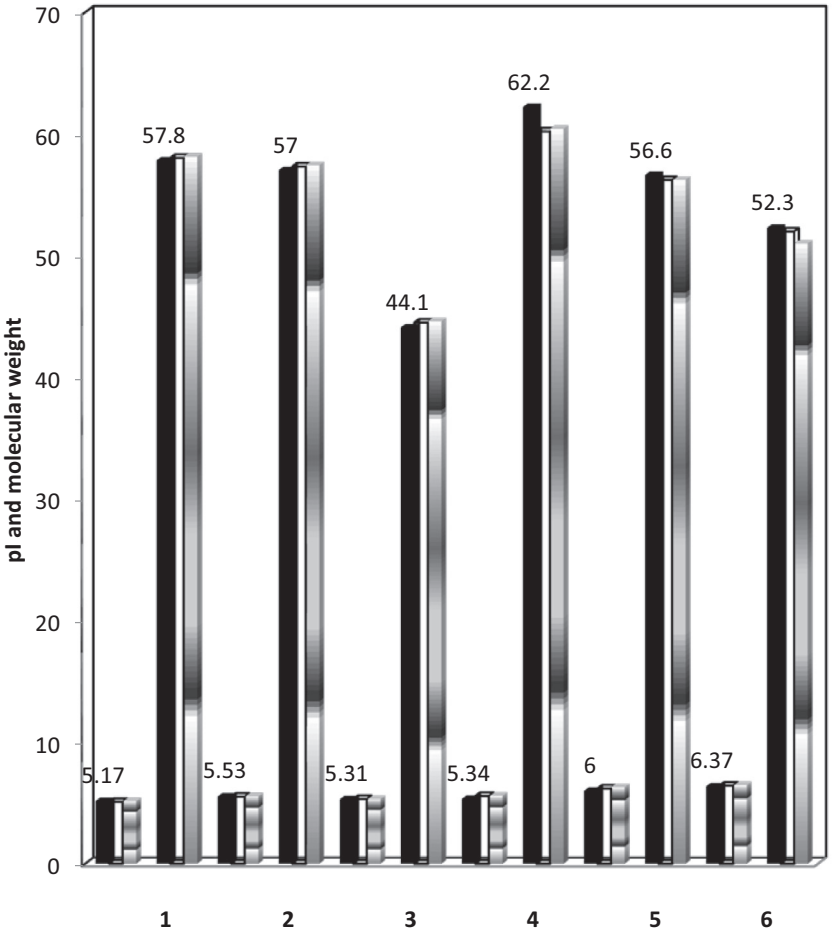

Figure 1 pI and molecular weight of over expressed proteins- (1) *N. muscorum*, (2) *N. muscorum* exposed to NaCl stress, (3) *N. muscorum* exposed to sucrose stress, (4) *B. japonicum*, (5) *B. japonicum* exposed to NaCl stress, (6) *B. japonicum* exposed to sucrose stress. Solid fills, Gradient fills, Gray fills.

non-ionic stress (osmotic stress) by the addition of sucrose. For the protein extraction cell was harvested and sample prepares for the 2 Dimension-Electrophoresis (2 DE) by the method of Natera et al. [17].

2.2. Protein isolation and extraction

The clarified supernatant was combined 1:1 with Tris-saturated phenol, pH 8.8 and after 30 min of gentle mixing at 4 °C, centrifuged at 8000 g for 10 min for both one-dimensional and two-dimensional electrophoretic analysis. The upper aqueous fraction was back-extracted once with an equal volume of Tris-saturated phenol and the lower phenolic fraction was extracted twice with an equal volume of 100 mM Tris–HCl, pH 8.8. Five volumes of MeOH containing 100 mM ammonium acetate and 10 mM DTT (chilled to -80 °C) were added to the pooled phenolic fractions and precipitated for 2 h at −80 °C. The solution now containing the precipitated proteins was centrifuged at 4000 g for 30 min. The resulting pellet was then resuspended and washed twice with several volumes of ice-cold MeOH containing 100 mM ammonium acetate and 10 mM DTT, then twice with several volumes of ice-cold acetone-H_2O (4:1, v/v) with 10 mM DTT. Between each wash and prior to centrifugation at 16,000*g* for 10 min, the solution was placed at −20 °C for 20 min. After the final aq. acetone wash, the protein pellet was dried at 25 °C for approximately 20 min to remove residual acetone, just prior to adding the IEF sample buffer. The pellet from the initial 400*g* centrifugation of the macerated nodules was dissolved in a minimal volume of 8.3 M urea, 2 M thiourea, 2% SDS and 100 mM DTT, vortexed heavily for several minutes and centrifuged at 8000 g for 10 min. The pellet was extracted three times using denaturing buffer with centrifugations of 8000 g for 10 min between extractions. The supernatants from each extraction were pooled and four volumes of ice-cold acetone–H_2O (4:1, v/v) containing 10 mM DTT were added for a 2 h precipitation at −20 °C. The protein precipitate was pelleted at 4000 g for 30 min and washed three times with several volumes of ice-cold acetone–H_2O (4:1, v/v) containing 10 mM DTT. Between each wash and prior to centrifugation at 16,000 g for 10 min, the solution was placed at −20 °C for 20 min. After the final wash, the protein pellet was dried at 25 °C for 20 min to remove residual acetone, then the IEF sample buffer was added prior to isoelectric focusing.

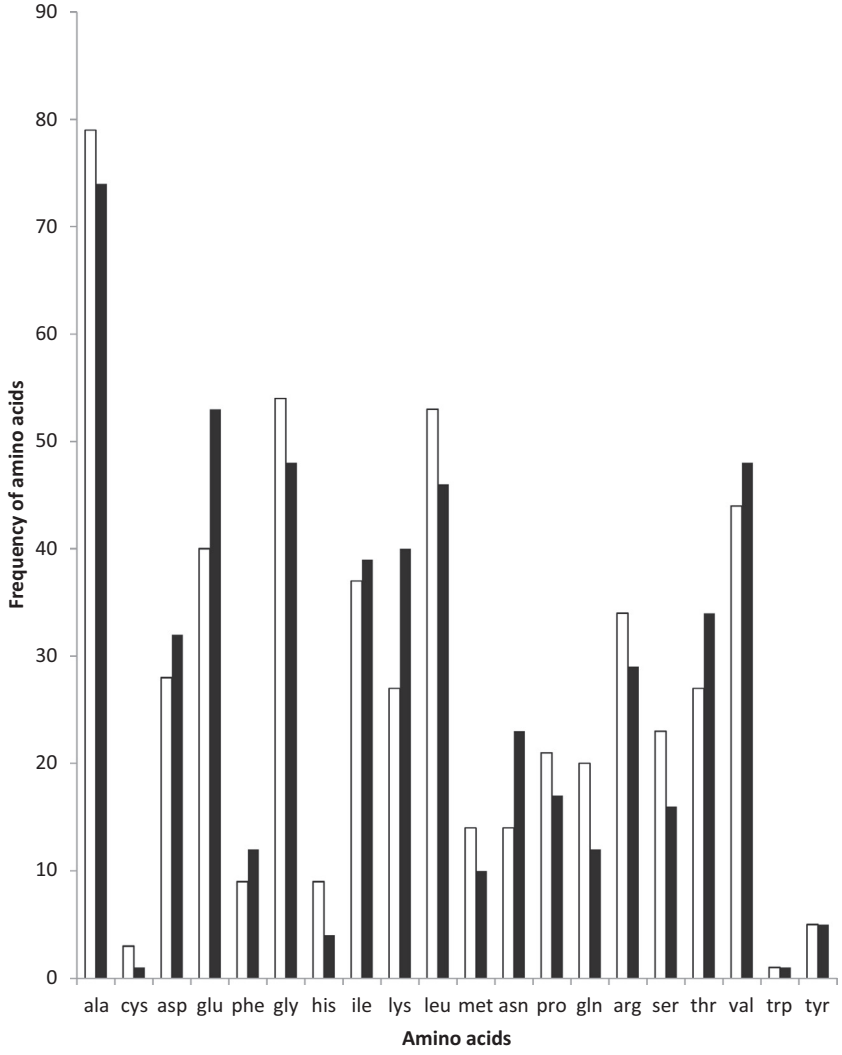

Figure 2 Amino acids frequencies of the GroEL chaperonin in *N. muscorum* (solid fills) and *B. japonicum* (gradient fills).

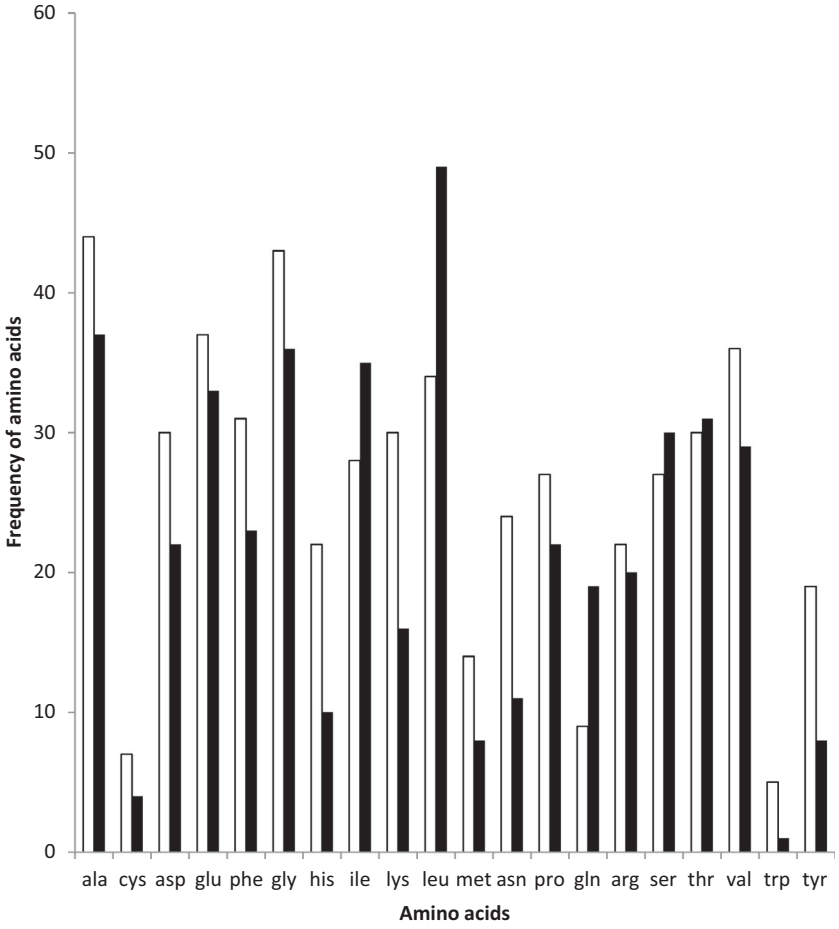

Figure 3 Amino acids frequencies of the Nitrogenase Mo-Fe protein in *N. muscorum* (solid fills) and *B. japonicum* (gradient fills).

2.3. Two-dimensional gel electrophoresis

2DE was performed with slight modification using PROTEAN II xi Cell (Bio-Rad). Isoelectric focusing was done in IPG strips (pH 3–10). This was given a pre-run for 190 Vh. The tubes were subsequently washed and loaded with samples containing 50 µg protein. Isoelectric focusing was conducted overnight for a total of 14,000 Vh. The tubes were extruded using an extrusion needle and equilibrated first in sample loading buffer containing 2% SDS, 50mMTris (pH 6.8), 6 Murea, 30% glycerol, 0.002% bromophenol blue and 1% DTT followed by 2.5% 2-iodoacetamide (instead of 1% DTT) for 15 min each. The gel was then loaded onto the second dimension having 12% T resolving gels and 4% T stacking gels (2 cm height). Electrophoresis was performed at 20 mA constant current for 12 h. Protein spots were characterized using a broad range molecular marker protein (Sigma) co-electrophoresed alongside the second dimension gel. After migration the gels were stained with coomassive brilliant blue R-250 (CBB).

2.4. Analysis of data

Protein spots were analyzed for differential expression patterns by using PDQuest software version 7.1 (Bio-Rad, USA). For the identification of the differentially expressed proteins, the analysis was carried out according to [18]. Spot intensities were calculated and normalized by determining the relative intensity of each spot (% volume) by dividing the intensity of each spot by the sum of the intensities of all spots on the corresponding gels. This work was done on commercial basis from The Center for Genomic Application (TCGA), New Delhi, India.

2 DE is a common method for analyzing differentially expressed proteins. The major drawback of this method is that it cannot be analyzed by poorly expressed proteins. Therefore, we have selected three proteins that are over expressed under the given stresses in both the species for further analysis.

3. Results and discussion

After matching the spot in 2D Gels, we obtain the different protein spot according to its molecular weight and pI against control (Fig. 1). Then, download the sequences in Fasta format from database (http://www.ncbi.nlm.nih.gov/) of these proteins. Arranged the sequences in notepad as input file for clastalw (http://www.ebi.ac.uk/Tools/msa/clustalw2/), submit the sequences and finds the cladogram file as .dnd, open it in MEGA 5.05, freely available software for phylogenetic analysis and display the tree.

The analysis of 2D images revealed that some unique spots were recorded under NaCl and sucrose caused stresses. In contrast, both the stresses induced some common stress proteins. In the present analysis we have selected only three protein molecules *viz*. GroEL chaperonin, nitrogenase Mo-Fe protein and argininosuccinate synthase for further

analysis. The availability of the complete amino acid sequence allows us to calculate amino acid frequencies of the over expressed proteins. The complete amino acid sequences of all the three proteins were obtained through NCBI data base (http://www.ncbi.nlm.nih.gov/). The amino acid sequences of the examined protein of both the organisms are given below:

B. japonicum GroEL Chaperonin
MPKIMLHDEA ARAALGRGVA KLAKAVRGTL
GPKGMNAIMD RPIGTPIVSR DGVSIAAEIE
LECPFENMGA QVLREVSKQT NDTAGDGTTT
ATVLADVLVQ DGLKCLAAGA NPVELVEGLE
LAVAETIKQL KRSAIAVQGS AGLRAVASIA
ANDAALGDMV AEAFERAGTH GIVAVEYGST
VQTTLEVIEG MAFERGYLSH HMVTDVERMQ
VVLDNPFILM TDHKIQSGEQ LAGVISLVEK
SGRPLLIIAE EVAPVVIMQL LARREKNNFK
VAAIHPPEFG HWRKAMLEDI AITTGGRVIS
VDLGGRLEKA ELQDLGAARQ VRISASKTLI
TAGAGDPDRI SARREQVMRQ YEAAPENIER
DKFQERIAKL SGGTAMILAG GATPVEQKRR
TQLIEDAINA TRAAIEEGIV PGGGFALLKA
APKLDELIKG LDGSVRQGAE LLQRSLSRPL
FHIASNAGLD AEAEVRKVAK GANGHGLDAR
NGASVDLVKA GIIDPVKVCY SAVRNAASVA
GLILTTQTLI AKKPDDYDPT AGPALGGGAELL

N. muscorum GroEL Chaperonin
MAKRIIYNEN ARRALERGID ILAEAVAVTL
GPKGRNVVLE KKFGAPQIVN DGVTIAKEIE
LEDHIENTGV ALIRQAASKT NDAAGDGTTT
ATVLAHAIVK EGLRNVAAGA NAILLKRGID
KATGFLVDRI KEHARPVEDS KSIAQVGSIS
AGNDDEVGQM IAEAMDKVGK EGVISLEEGK
SVTTELEITE GMRFDKGYIS PYFATDPERM
EAIFDEPFLL LTDKKIALVQ DLVPVLEQVA
RAGRPLVIIA EDIEKEALAT LVVNRLRGVL
NVAAVKAPGF GDRRKAMLED IAILTGGQLI
TEDAGLKLEN TKLESLGKAR RITITKDSTT
IVAEGNDVAV KGRVEQIRRQ MEETESSYDK
EKLQERLAKL SGGVAVVKVG AATETEMKDK
KLRLEDAINA TKAAVEEGIV PGGGTTLAHL
TPELEVWANS NLKDEELTGA LIVARALPAP
LKRIAENAGQ NGAVIAERVK EKAFNVGFNA
ATNEFVDMFE AGIVDPAKVT RSALQNAASI
AGMVLTTECI VVDKPEPKDN APAGAGAGGG
DFDY

B. japonicum Nitrogenase Mo-Fe protein *nifk*
MTQSAEHVLD HFELFRGPEY QQMLANKKKM
FENPRDPAEV ERIREWAKTP EYREKNFARE
ALTVNPAKAC QPLGAVFVAV GFEKTLPFVH
GSQGCVAYYR SHLSRHFKEP SSCVSSSMTE
DAAVFGGLNN MIDGLANAYN MYKPKMIAVS
TTCMAEVIGD DLNAFIKTSK EKGSVPAEYD
VPFAHTPAFV GSHVTGYDNA MKGILDHFWD
GKAGTAPKLE RVPNEKINFI GGFDGYTVGN
IREVKHIFEE MGIEYTILGD NSNVFDTPTD
GEFRMYEGGT TLEDAANAVH AKATISMQHY
CTEKTLPFIA NHGQETVSFH HPVGVSGTDA
FVMALSRISG KEIPESLAVE RGRLVDAIAD
SSAHIHGKKF AIYGDPDLCL GLASFLLELG
AEPTHVLATN GNKEWAERVQ AVFDASPFGK
NCHVYPGKDL WHMRSLLFTE PVDFLIGNTY
GKYLERDTGT PLIRIGFPIF DRHHKHRYPV
WGYQGGLNVL VTILDKIFDE IDRNTNVPAK
SDYSFDIIR

N. muscorum Nitrogenase Mo-Fe protein
MAIVTLPNKS VAVNPLKQSQ ALGASLAFLG
LKGMIPLFHG SQGCTAFAKV VLVRHFREAI
PLATTAMTEV TTILGGEDNI EQAILTLVEK SSPEIIGLCS
TGLTETRGDD IERFLKDIRD
RHPEISHLPI VFAPTPDFKG ALQDGFAAAV
ESIVQEIPQP GTTRSEQVTI LAGSAFTPGD
LQEIKEIVTA FGLVPIFVPD IGASLDGHLD EEYSSVTTSG
TTVKQLQEVG CSAFTIALGE
SMRGAARILE DRFNIPYEVF SELTGLEPVD
EFIQALAILS SNSVPEKYCR QRRQLQDAML
DTHFYFGAKR ISLALEPDLL WSMVKFLQSM
GTQIHAAVTT TRSPLLEQLP IKSVTIGDLE
DFEQLAVESD LLIGNSNLAA IAKRLSIPHY
RLGIPIYDRL GNGHYTKVGY RGSMEVLFGI
GNLFIDAEEA RVKNFDENFV TGHG

B. japonicum Argininosuccinate synthase
MTTILKSLPK GEKVGIAFSG GLDTSAALLW
MKQKGARCYA YTANLGQPDE ADYNEIPRKA
QEFGAEKAVL VDCRTQLVHE GIAAIQSGAF
HISTGGITYF NTTPLGRAVT GTMLVAAMKE
DGVNIWGDGS TFKGNDIERF YRYGLLTNPS
LRIYKPWLDQ QFIDELGGRA EMSAFMTAQG
FAYKMSAEKA YSTDSNLLGA THEAKDLESL
DSGIKIVNPI MGVPFWREDC NVKAEKVVVR
FEEGQPTALN GQTFTDPVAL FLEANAIGGR
HGLGMSDQIE NRIIEAKSRG IYEAPGMALL
HIAYERLVTG IHNEDTIEQY RISGMRLGRL
LYQGRWFDSQ ALMLRETAQR WVARAVTGEV
TLELRRGNDY SILNTESPNL TYAPERLSME
KVEDAAFTPA DRIGQLTMRN LDIADTRTKL
KLYTDTGLLS GSEGSQIFRL ENDKG

N. muscorum Argininosuccinate synthase
MGRAKKVVLA YSGGVDTSVC IPYLKQEWGV
EEVITLAADL GQGDELEPIR EKALKSGASE
SLVADVKDSF VKDYAFGAIQ ANALYENRYP
LGTALARPLI AKILVETAEK YGADAIAHGC
TGKGNDQVRF DVSVTALNPN LKILAPAREW
GMSREETIAY GEKFGIPSPV KKSSPYSIDK
NLLGRSIEAG LLEDPSFEPP EEIYEMTKAI ADTPNEPEYI
KIGFHGGIPT TLNGIAKQPV
ELIEELNQLV GNHGVGRIDM IENRLVGIKS
REIYESPAML VLIQAHRDLE SLTLTADVTH
YKRGIEETYS QIVYNGLWYS PLKVALDAFI
QKTQERVSGT VRVKLFKGNS TIVGRSSDSS
LYTPDLATYG ADDQFDHKAA EGFIYVWGLP
TRIWSRQIKS

The amino acid sequences were further characterized in terms of amino acid composition and frequency. The comparative frequency of amino acid composition of GroEL chaperonin, nitrogenase Mo-Fe protein and argininosuccinate synthase is given in Figs. 2–4. When we compared the amino acid frequency of all the three proteins it has been observed that all the three proteins contain maximum amounts of alanine, glycine and valine (amino acids with abundant frequency) An increase in glycine content is supported by the finding of Srivastava et al. [18], they reported salt-induced increase in glycine content in *Anabaena doliolum*. On the other hand amino acids such as cysteine and tryptophan occurred in less frequency. All the most abundant amino acids observed in the present investigation were believed to have been common in pre-biotic environment [19]. This type of proteomic

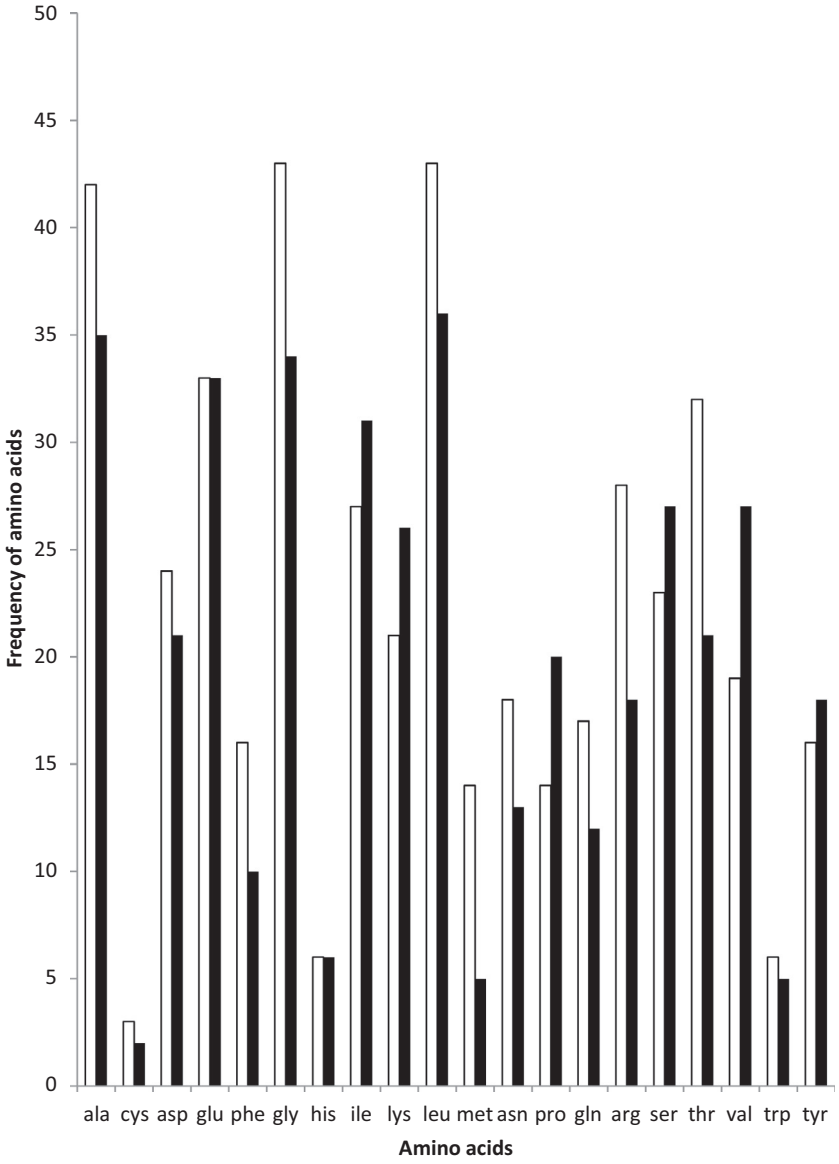

Figure 4 Amino acids frequencies of the Argininosuccinate synthase in *N. muscorum* (solid fills) and *B. japonicum* (gradient fills).

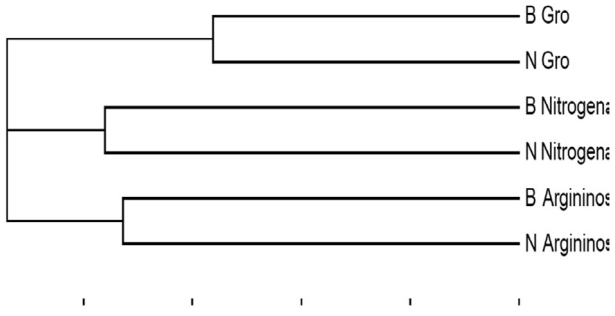

Figure 5 A cladogram showing relationship among three over expressed proteins (B- *B. japonicum* and N- *N. muscorum*).

comparison would help in tracing phylogenetic relationship between protein families. A similar type of study on protein evolution and its relation to genetic code has been conducted by Brooks et al. [20].

Protein synthesis is an energy requiring process and the biosynthetic cost of the protein molecules depends upon the amino acid composition. In natural ecosystems living organisms selected cheaper amino acids to synthesize protein molecules for their optimum growth and survival. Thus, the biosynthetic cost of amino acids also plays an important role in the evolution of amino acids/proteins. In *Saccharomyces* it has been reported that expensive amino acids may only be synthesized when they have specific structural or functional roles in protein sequence [21]. In the examined proteins it has been assessed that most abundant amino acids are energetically cheaper to produce on the other hand less abundant amino acids are energetically expensive. This conclusion is based on the studies on comparative cost of amino acids biosynthesis in different organisms [22–25].

Since the composition and frequency of different amino acids were found to show similarity between the two strains, so in the next series of analysis we have compared amino acid sequence by preparing cladogram (Fig. 5). We used PAM for

the preparation of cladogram. The analysis of all the three protein molecules through cladogram suggested that GroEL chaperonin were evolutionarily closer to each other. On the contrary nitrogenase Mo-Fe proteins were evolutionary distinct from each other. The protein argininosuccinate synthase is also showing distinct evolution but less than nitrogenase Mo-Fe protein.

4. Conclusion

The above observation implied that abundant amino acids/proteins were believed to have been common in the prebiotic environment. Further it is also concluded that the above examined amino acids/proteins were originated simultaneously during the course of evolution.

Acknowledgements

S. Bhargava and V. Kaithwas are thankful to the University Grants Commission, New Delhi for financial support. K. Bhardwaj acknowledges Vimta Lab Hyderbad for 2D analysis and PDQuest analysis. D. Gupta is thankful to Head Department of Biotechnology and Bioinformatics for necessary facilities.

References

[1] A. Latifi, M. Ruiz, C. Zhang, F.E.M.S. Microbiol, Review 33 (2009) 258–278.
[2] M.E. Feder, Am. Zool. 39 (2009) 857–864.
[3] P.H. Moisander, E. McClinton, H.W. Paerl, Microbiol. Ecol. 43 (2002) 432–442.
[4] N. Murata, I. Suzuki, J. Exp. Bot. 57 (2006) 235–247.
[5] R.S. Ullers, D. Ang, F. Schwager, P. Geogopoulos, Pro. Natl. Acad. Sci. U.S.A. 104 (2007) 3101–3106.
[6] K.R. Diller, Annu. Rev. Biomed. Eng. 8 (2006) 403–424.
[7] A.J. Macario, M. Lange, B.K. Ahring, E.C. De Macario, Microbiol. Mol. Biol. Rev. 63 (1999) 923–967.
[8] D.S. Latchman, in: Stress Proteins, Springer, Berlin, 1999, p. 422.
[9] K.C. Kregel, J. Appl. Physiol. 92 (2002) (2002) 2177–2186.
[10] M. Schroder, R.J. Kaufman, J. FMMR 569 (2005) 29–63.
[11] K. Kvint, L. Nachin, A. Diez, T. Nyström, Curr. Opin. Microbiol. 6 (2003) 140–145.
[12] Q. Ma, T.K. Wood, Biochem. Biophys. Res. Commun. 410 (2011) 846–851.
[13] D. Rangel, World J. Microbiol. Biotechnol. 27 (2011) 1281–1296.
[14] D. Kultz, J. Exp. Biol. 206 (2003) 3119–3124.
[15] G.C. Gerloff, G.P. Fitzgerald, F. Skoog, Am. J. Bot. 37 (1950) 216–218.
[16] M.J. Sadowsky, R.E. Tully, P.B. Cregan, H.H. Keyser, Appl. Environ. Microbiol. 53 (1987) 2624–2630.
[17] S.H.A. Natera, N. Guerreiro, M.A. Djordjevic, Mol. Plant-Microbe Interact. 13 (2000) 995–1009.
[18] A.K. Srivastava, P. Bhargava, R. Thapar, L.C. Rai, Environ. Exp. Bot. 64 (2008) 49–57.
[19] S.L. Miller, Cold Spring Harbor Symp. Quant. Biol. 52 (1987) 17–27.
[20] D.J. Brooks, J.R. Fresco, A.M. Lesk, M. Singh, Mol. Biol. 19 (2002) 1645–1655.
[21] M.D. Barton, D. Delneri, S.G. Oliver, M. Rattray, et al, PLoS One 5 (2010) 11935–11942.
[22] H. Akashi, Pro. Natl. Acad. Sci. U.S.A. 99 (2002) 3695–3700.
[23] C.L. Craig, R.S. Weber, Mol. Biol. Evol. 15 (1998) 774–776.
[24] A. Wagner, Mol. Biol. Evol. 22 (2005) 1365–1374.
[25] H. Seligmann, J. Mol. Evol. 56 (2003) 151–161.