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In silico Approach to Elucidate Factors Associated with GH1 β-Glucosidase Thermostability

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Abstract

β-Glucosidase is a class of hydrolytic enzymes that catalyzes the removal of the non-reducing β-D-glucosyl unit from various disaccharides and substituted β-D-glucosides. β-Glucosidase belongs to Glycoside Hydrolase (GH) families 1 and 3 and potentially has many biotechnological applications with thermostable enzymes are preferred over mesophilic homologs in different applications. In the present work, a comparative analysis of physicochemical properties and amino acids composition of 60 (20 mesophilic, 20 thermophilic and 20 hyperthermophilic) β-glucosidases were performed. Multiple sequence alignment and phylogenetic tree analysis were constructed. Analysis of Variance (ANOVA) showed that several physicochemical properties including molecular weight, isoelectric point, number of positively charged amino acids, and extinction coefficient are statistically different among β-glucosidases groups (*P*<0.05). The analysis also showed that content of amino acids Asp, Gln, Cys, His, and Thr is significantly higher in mesophilic enzymes whereas that of Glu, Lys, Tyr, and Trp is higher in thermo- and hyperthermostable homologs (*P*<0.05). Overall, nonpolar amino acids were the most abundant amino acids group in β-glucosidase with no significant difference among meso-, thermo-, and hyperthermophilic enzymes. Conversely, the content of polar amino acids is statistically higher (*P*<0.05) in mesophilic enzymes whereas that of charged and aromatic amino acids is significantly higher (*P*<0.05) in thermo- and hyperthermophilic counterparts. Finally, multiple regression analysis showed that both polar and aromatic amino acids contribute significantly (*P<0.05*) to the thermostability. Optimal temperature variation of 53% could be explained by these two groups of amino acids. In conclusion, several amino acids appear to contribute to the thermostability of β-glucosidases and the findings from this study should pave the road toward a better understanding of thermostability of β-glucosidases and protein engineering.

Keywords: β-Glucosidase, thermostability, amino acid composition, physicochemical properties, ANOVA, Regression.

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INTRODUCTION

β-Glucosidase (BGL) is a heterogeneous group of hydrolytic enzymes that catalyzes the removal of the non-reducing β-D-glucosyl terminal unit from a variety of disaccharides, alkyl-β-D-glucosides, aryl-β-D-glucosides and short oligosaccharides[1,2]. BGLs have found tremendous applications in various biotechnological industries mainly biofuel production, aroma and flavor enhancement, nutritional isoflavone hydrolysis, cassava detoxification, paper deinking, and synthesis of various oligosaccharides and substituted-β-D-glycosides[2,3]. Hence BGLs have attracted the interest of researchers of this field in the last decade. Additionally, many applications of BGL such as biofuel production require enzymes with exceptional properties such as increased catalytic efficiency, high thermostability, and glucose tolerance[4,5]. BGLs such as GH 3 BGLs from fungi are sensitive to glucose. However, several reported GH 1 BGLs exhibit excellent glucose tolerance[6-10]. Thermostability of BGL from GH 1 family is low and the search for thermostable enzymes with glucose tolerance is an important goal of ongoing researches. In this context, on one hand, thermostable enzymes can be obtained through isolation of novel microbes capable of producing thermostable enzymes; which is tedious, time-consuming and cost-intensive approach[11]. On the other hand, the application of protein engineering principles to design and synthesize thermostable proteins from their mesophilic homologs is the approach of choice toward the development of industrially convenient catalysts[3,12].

Elucidation of factors contributing to protein thermostability is the first crucial step for successful protein engineering and catalysts designing for the conversion of mesophilic enzymes to thermophilic counterparts. Several workers of the field have identified some attributes contributing to protein thermostability including hydrophobicity and compactness[13-15], shortening of loops[16-18], decreased occurrence of thermolabile residues such as Gln, Cys, and Ser[13], high content of aromatic amino acids[19], high helical content[20], increased polar surface area[21], hydrogen bonding and electrostatic interactions[13,20], high frequency of proline occurrence[22], and high disulfide bonds[13,23,24]. These factors can be determined experimentally or through the analysis of protein sequences and structures using robust computational biology and bioinformatics tools; known as *in silico* approach. This approach is more attractive because it is cost effective and enables comparison and analysis of large datasets of protein. Detailed comparative analysis of physicochemical properties and amino acids composition of mesophilic, thermophilic, and hyperthermophilic BGLs is lacking. The present study aimed to compare the physicochemical properties and amino acids composition of mesophilic, thermophilic and hyperthermophilic BGLs from GH 1 in an attempt to identify attributes associated with enhanced thermostability of BGLs which may pave the way toward future engineering of BGL.

MATERIALS AND METHODS

Data Collection

Different literature databases (e.g., PubMed, ScienceDirect, Springer, Google Scholar) were searched for publications regarding GH1 BGLs. Publications were downloaded and screened for information regarding source organisms, life domain, optimal temperature of enzyme activity and Genbank or UniProt ID (if reported). Only GH1 BGLs which have been characterized for substrate specificity and optimal temperature were selected for further analysis. Protein sequences were retrieved from UniProt (https://www.uniprot.org/) in FASTA format for analysis. Based on reported optimal temperature of enzyme activity, these enzymes were classified into three groups: 1) mesophilic with an optimal temperature between 25-45°C (M-BGLs), 2) thermophilic with an optimal temperature between 50-75°C (T-BGLs), and 3) hyperthermophilic with an optimal activity above 75°C (HT-BGLs).

Deduction of Physicochemical Properties and Amino Acid Compositions

Protein sequences were predicted for the presence of signal peptide using Signal P 4.1 server (http://www.cbs.dtu.dk/services/SignalP/)[25], and localization using LocTree 3 and PSORTb (https://rostlab.org/services/loctree3/ and http://www.psort.org/psortb/)[26,27]. Various physicochemical properties and amino acids composition of protein sequences were also predicted using the

EXPASY tool ProtParam (https://web.expasy.org/protparam/)[28,29]. The physicochemical properties predicted include numbers of amino acids, molecular weight (MW), Isoelectric Points (PI), number of negatively (Asp and Glu) and positively (Lys and Arg) charged residues, extinction coefficient, Instability Index (II), Aliphatic Index (AI) and Grand Average of Hydropathicity (GRAVY).

Sequence Alignment and Phylogenetic Tree Construction

The retrieved sequence of M-BGLs, T-BGLs, and HT-BGLs were aligned using muscle tool for multiple sequence alignment (MSA) at EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/muscle/)[30]. The alignment was retrieved in FASTA format and edited by BoxShade server (https://embnet.vital-it.ch/software/BOX_form.html)[31]. Further, MSA was submitted to Phylogeny online tool (http://www.phylogeny.fr/) to construct a phylogenetic tree of selected BGLs sequences[32] using the defualt setting (maximum likelihood method, WAG substitution model, bootstrap 16).

Statistical Analysis and Significance Inference

Graphpad Prism 5 was used for calculating statistical parameters of physicochemical properties and amino acids compositions for M-BGLs, T-BGLs, and HT-BGLs. First, analysis of variance (ANOVA) was carried out to find whether there is a significant difference in the means of the parameters of M-BGLs, T-BGLs, and HT-BGLs. The null hypothesis states that there is no significant difference in the means of physicochemical properties and amino acid composition between M-BGLs, T-BGLs, and HT-BGLs. The confidence interval for significance was 95% and P-value <0.05 was considered significant. Next, where ANOVA detected a significant difference, post hoc Tukey's test was used for multiple comparisons of the means of two groups. Finally, attributes showed a significant difference between M-BGLs, T-BGLs, and HT-BGLs and correlated with the optimal temperature of BGLs activity were used for multiple linear regression analysis to construct a model for optimal temperature prediction from amino acids compositions.

RESULTS AND DISCUSSION

Multiple Sequence Alignment and Phylogenetic Tree Construction

Total sixty GH1 BGL sequences for which experimental optimal temperature has been determined (20 M-BGLs, 20 T-BGLs, and 20 HT-

Table 1. Mesophilic GH1 β-Glucosidase with UniProt ID and reported optimal temperature

Enzyme ID	UniProt ID	Source organism	Domain	Optima T (°C)	Reference
M-BGL01	K0A8J9	*Exiguobacterium antarcticum* B7	Bacteria	30	[33]
M-BGL02	O93785	*Hypocrea jecorina*	Fungi	40	[34]
M-BGL03	A1D6G3	*Neosartorya fischeri* NRRL181	Fungi	40	[35]
M-BGL04	F1JZ12	*Sphingomonas* sp. strain 2F2	Bacteria	37	[36]
M-BGL05	B9V8P5	*Micrococcus antarcticus*	Bacteria	25	[37]
M-BGL06	A0A1S5SJM8	Unculturable bacterium	Bacteria	40	[38]
M-BGL07	D5KX75	Unculturable bacterium	Bacteria	40	[8]
M-BGL08	I6YQJ8	Unculturable bacterium	Bacteria	40	[39]
M-BGL09	E6TUY6	*Bacillus cellulosilyticus*	Bacteria	40	[40]
M-BGL10	I6TNE2	*Weissella cibaria*	Bacteria	45	[41]
M-BGL11	Q9F3B7	*Streptomyces coelicolor* A3	Bacteria	35	[42]
M-BGL12	Q9K440	*Streptomyces coelicolor* A3	Bacteria	35	[42]
M-BGL13	D0VLH9	*Exiguobacterium oxidotolerans*	Bacteria	35	[43]
M-BGL14	J9XU85	*Bifidobacterium lactis*	Bacteria	38	[44]
M-BGL15	B8HAF9	*Arthrobacter chlorophenolicus*	Bacteria	37	[45]
M-BGL16	A0A1L3HS62	Uncultured bacterium	Bacteria	37	[46]
M-BGL17	A0A2I2LGB3	Uncultured bacterium	Bacteria	40	[47]
M-BGL18	A0A1W6I0S4	Uncultured bacterium[a]	Bacteria	38	[48]
M-BGL19	A6W3B1	*Marinomonas* MWYL1	Bacteria	40	[49]
M-BGL20	M4I6Y9	*Lactococcus* sp. FSJ4	Bacteria	40	[50]

[a]Amino acids at position 1-18 were predicted as signal sequence and removed.

BGLs; Tables 1-3, respectively) were retrieved from the UniProt database. Multiple Sequence Alignment (MSA) analysis revealed that several amino acids motifs are conserved among all GH1 BGLs. β-Glucosidase is a single polypeptide protein that folds to form a GH1 classical (β/α)8 TIM barrel structure comprised of eight α-helices and eight β-strands linked by short loops. GH1 BGL utilizes two key glutamic acid residues as a general acid/base catalyst and nucleophile[87]. MSA

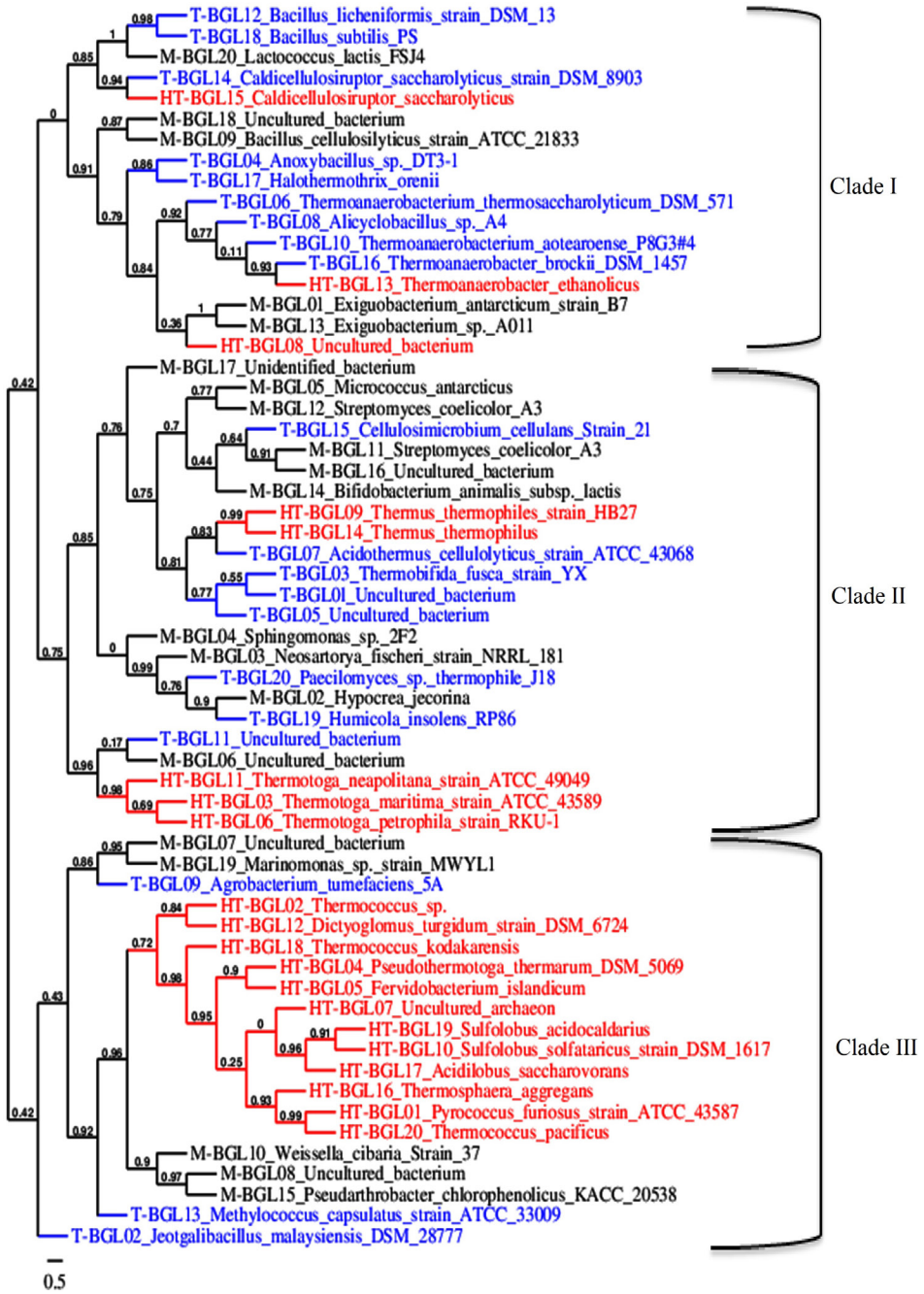

Fig. 1. Phylogenetic tree of mesophilic (Black, M-BGL), thermophilic (blue, T-BGL) and hyperthermophilic (red, HT-BGL) β-glucosidases from bacteria, archaea, and fungi. This phylogenetic tree was constructed using Phylogeny tool.

showed the conservation of both Glu residues in all BGLs regardless of optimal temperature. The first Glu residue is the general acid/base conserved at position 166 (for BGL from *Humicola insolens* (HiBGL) as reference, see supplementary data S. Fig. 1) at conserved motif TXNEP (Thr-X-Asn-Glu-Pro) and the second Glu residue is the nucleophile conserved at position 377 in consensus sequence TENG (Thr-Glu-Asn-Gly)[88]. The active site is located at C-terminal of the barrel and is made up of two subsites namely glycon binding site (subsite -1) and aglycon binding site (subsite +1). The catalytic acid/base is located at the C-terminal of β-strand 4 and the nucleophile at the C-terminal of the β-strand 7[88]. In HiBGL, glycon binding site (subsite -1) lies at the bottom of the barrel with Gln17, His120, Trp121, Asn165, Tyr308, Trp427, Glu434, Trp435 and Phe443 residues[65]. MSA showed that these residues are conserved throughout BGL evolutionary history and the side chains of which interact with glycon moiety through both hydrogen and hydrophobic bonds. Conversely, aglycon binding site (subsite +1) is less conserved and is determined by Thr177, Tyr179, Phe325, Leu326, Thr331, Phe333 and Phe348 (in HiBGL numbering) which function as gatekeepers and explain the aglycone broad substrate specificity exhibited by this enzyme[89]. Moreover, Trp 168 and Leu173 were found to be responsible for glucose tolerance[90] and MSA showed that these two residues are conserved among high glucose-tolerant BGLs. The aglycon appeared to anchor by hydrophobic contacts and water-mediated polar bonds[91]. In contrarily, there are few studies on amino acids/motifs associated with thermostability of BGLs. Tamaki *et al.* 2014 employed Statistical Coupling Analysis (SCA) to identify several amino acids related to the thermostability of BGL from *Spodoptera frugiperda* (Sfβgly) (corresponding to Arg27, Pro39, Trp121, Pro167, His211, Pro266, Pro286, Trp435 and Phe443 in HiBGL numbering)[92]. MSA demonstrated that these residues are conserved and the majority of which are proline or positively charged amino acids. Additionally, these residues appeared to be distributed in the loop segments of BGL whereas, amino acids related to enzyme activity are mainly concentrated around α-helices and β-strands[92]. Altogether these

Table 2. Thermophilic GH 1 β-Glucosidase with UniProt ID and reported optimal temperature

Enzyme ID	UniProt ID	Source	Domain	Optima T (°C)	Reference
T-BGL01	HV538882.1	Uncultured bacterium	Bacteria	75	[51]
T-BGL02	A0A0B5ARU7	*Jeotgalibacillus malaysiensis*	Bacteria	65	[52]
T-BGL03	Q47RE2	*Thermobifida fusca*	Bacteria	60	[53]
T-BGL04	M5QUM2	*Anoxybacillus sp. DT3-1*	Bacteria	70	[10]
T-BGL05	K4I4U1	Uncultured bacterium	Bacteria	50	[54]
T-BGL06	D9TR57	*Thermoanaerobacterium thermosaccharolyticum*	Bacteria	70	[55]
T-BGL07	A0LR48	*Acidothermus cellulolyticus*	Bacteria	70	[56]
T-BGL08	A0A220YLM5	*Alicyclobacillus sp.*	Bacteria	55	[6]
T-BGL09	H0HC94	*Agrobacterium tumefaciens 5A*	Bacteria	52	[57]
T-BGL10	A0A0H4NXH8	*Thermoanaerobacterium aotearoense*	Bacteria	60	[9]
T-BGL11	W0LHR5	Uncultured bacterium	Bacteria	60	[58]
T-BGL12	Q65D37	*Bacillus licheniformis*	Bacteria	50	[59]
T-BGL13	Q608B9	*Methylococcus capsulatus*	Bacteria	70	[60]
T-BGL14	A4XIG7	*Caldicellulosiruptor saccharolyticus*	Bacteria	70	[61]
T-BGL15	A0A220IP58	*Cellulosimicrobium cellulans*	Bacteria	55	[62]
T-BGL16	*Q60026*	*Thermoanaerobacter brockii*	Bacteria	75	[63]
T-BGL17	*B8CYA8*	*Halothermothrix orenii*	Bacteria	70	[64]
T-BGL18	I3QIG4	*Bacillus subtilis*	Bacteria	60	[7]
T-BGL19	A0A076JRL8	*Humicola insolens* RP86	Fungi	60	[65]
T-BGL20	H8XVY6	*Paecilomyces thermophila*	Fungi	55	[66]

residues represent a hotspot for BGL engineering in future. However, further studies to identify more amino acids variants and motifs related to the thermostability in M-, T-, and HT-BGL may be required.

Multiple sequence alignment was used to construct a phylogenetic tree to visualize the evolutionary relationship between M-, T-, and HT-BGLs. BGLs were clustered into three major clades (Fig. 1). Clade I was dominated by T-BGLs (9 sequences, 52.9%) followed by M-BGLs (5, 29.4%) and HT-BGLs (3, 17.7%). Clade II was dominated by M-BGLs (10, 43.5%) followed by T-BGLs (8, 34.8%) and HT-BGLs (5, 21.7%). Both mesophilic and thermophilic fungal BGLs analyzed were clustered together in this clade suggesting their bacterial origin. Clade III was dominated by HT-BGLs from both bacteria and archaea (12, 60%) followed by M-BGLs (5, 25%) and thermostable BGLs (3, 15%). Clustering of mesophilic, thermophilic and hyperthermophilic BGLs together indicates the existence of structural and functional similarities. The clustering of mesophilic and thermophilic protein is in agreement with previous reports[93,94]. Similarly, HT-BGLs from both archaea and bacteria were also clustered together in clade III indicating the structural similarity among them. Archaeal and bacterial proteins have been clustered together in several phylogenetic tree analyses[95,96]. This is because many proteins distinguishing these two domains belong to information processing proteins such as DNA replicating enzymes, and transcription and translation associated protein[97].

Comparative Analysis of Physicochemical Properties

All GH1 BGLs appear to lack of signal peptide and to localize in the cytoplasm except M-BGL-18 which was predicted to have 18 residues and to localize in the periplasm. GH 1 BGLs are known to be localized in the cytoplasm[3]. Statistical analysis (ANOVA and followed by Tukey test) demonstrated that MW of HT-BGLs is significantly higher than M-BGLs or T-BGL (P<0.05, Table 4). Increase in the MW of HT-BGLs could be attributed to the higher content of larger amino acids such as Lys, Tyr and Trp and lower content of smaller amino acids such as Gly, Gln, and Cys in HT-BGLs[98,99]. Similarly, PI is significantly higher in HT-BGLs than M-BGLs and T-BGLs (P<0.05, Table 4). A similar finding was reported for thermostable nitrilase over their mesophilic counterparts[95]. PI indicates the pH at which the protein has an

Table 3. Hyperthermophilic GH1 β-Glucosidase with UniProt ID and reported optimal temperature

Enzyme ID	UniProt ID	Source organism	Domain	Optima T (°C)	Reference
HT-BGL01	E7FHY4	*Pyrococcus furiosus*	Archaea	100	[67]
HT-BGL02	O08324	*Thermococcus sp.*	Archaea	78	[68]
HT-BGL03	Q08638	*Thermotoga maritima*	Bacteria	95	[69]
HT-BGL04	F7YX70	*Thermotoga thermarum*	Bacteria	90	[70]
HT-BGL05	G8YZD7	*Fervidobacterium islandicum*	Bacteria	90	[71]
HT-BGL06	A5IL97	*Thermotoga petrophila*	Bacteria	80	[72]
HT-BGL07	W8W3B8	Uncultured bacterium	Achaea	90	[73]
HT-BGL08	A0A0A6ZH67	Uncultured bacterium	Bacteria	90	[74]
HT-BGL09	Q746L1	*Thermus thermophiles* HB27	Bacteria	88	[75]
HT-BGL10	P22498	*Sulfolobus solfataricus*	Archaea	90	[76]
HT-BGL11	B9K7M5	*Thermotoga neapolitana*	Bacteria	95	[77]
HT-BGL12	B8E1X9	*Dictyoglomus turgidum*	Bacteria	80	[78]
HT-BGL13	D3Y2V4	*Thermoanaerobacter ethanolicus*	*Bacteria*	*80*	*[79]*
HT-BGL14	A8WAC9	*Thermus thermophiles* HJ6	Bacteria	90	[80]
HT-BGL15	P10482	*Caldocellum saccharolyticum*	Bacteria	85	[81]
HT-BGL16	Q9YGA8	*Thermosphaera aggregans*	Archaea	85	[82]
HT-BGL17	D9PZ08	*Acidilobus saccharovorans*	Archaea	93	[83]
HT-BGL18	Q9YGB8	*Pyrococcus kodakaraensis*	Archaea	100	[84]
HT-BGL19	P14288	*Sulfolobus acidocaldarius*	Archaea	85	[85]
HT-BGL20	A0A0A7RBQ4	*Thermococcus pacificus P-4*	Archaea	75	[86]

Table 4. Statistical analysis of physicochemical properties of GH1 β-glucosidases

Physicochemical properties	Average ± SD			ANOVA Statistics	Tukey's multiple comparison test, significant?		
	M-BG	T-BG	HT-BG	F Value P Value	M vs T	M vs HT	T vs HT[a]
No. of amino acid residues	455.1 ± 21.71	462.35 ± 15.27	461.35 ± 25.81	0.676 0.51	No	No	No
Molecular Weight (Da)	51390.9 ± 2223.73	52518.51 ± 1365.76	53321.49 ± 3079.78	3.463 0.038	No	Yes	No
Theoretical PI	5.28 ± 0.81	5.27 ± 0.31	5.80 ± 0.42	5.882 0.005	No	Yes	Yes
No. of negatively charged residue (Asp+Glu)	62.05 ± 8.65	66.55 ± 5.58	63.75 ± 6.18	2.15 0.126	No	No	No
No. of positively charged residue (Arg+Lys)	41.8 ± 5.47	47.45 ± 7.28	53.75 ± 4.87	20.11 0.000	Yes	Yes	Yes
Extinction Coefficients	104913 ± 14154.08	111164.75 ± 10943.43	124925.75 ± 14061.23	12.146 0.000	No	Yes	Yes
Instability Index II	32.76 ± 5.25	32.11 ± 4.20	32.57 ± 5.97	0.084 0.92	No	No	No
Aliphatic Index AI	77.71 ± 6.74	77.75 ± 4.44	79.23 ± 4.66	0.577 0.565	No	No	Yes
Grand average of hydropathicity (GRAVY)	-0.37 ± 0.13	-0.4152 ± 0.11	-0.41675 ± 0.1	1.171 0.318	No	No	No

[a] M for M-BGL, T for T-BGL, and HT for HT-BGL.

equal number of positive and negative charges. However, a study on a set of 310 proteins failed to correlate pH or temperature stability with PI[100,101]. Additionally, the analysis showed that numbers of positively charged amino acids (Lys and Arg) are higher in HT-BGLs than M-BGLs and T-BGLs (P< 0.05, Table 4). Increased content of positively charged amino acids in thermostable BGLs can be postulated to involve in salt bridge formations and thus enhancing protein thermostability[102-105]. Indeed, there is experimental evidence showing that the redesigning of salt bridge significantly enhanced BGL thermostability[106]. Finally, the extinction coefficient is also statistically higher in HT-BGLs than M-BGLs and T-BGLs (P<0.05, Table 4). Extinction coefficient reflects aromatic amino acids content (Phe, Tyr, and Trp) which in turn appears to enhance protein thermostability through increasing protein hydrophobicity and packing[106]. Conversely, number of negatively charged amino acids, AI, II, and GRAVY did not show any statistical difference in their means among M-, T-, and HT-BGLs. Similar findings have been reported for nitrilase/cyanide hydratase family from mesophilic, thermophilic, and hyperthermophilic bacteria[95] and serine protease from mesophilic and thermophilic microorganisms[107]. AI indicates the relative volume occupied by the side chain of hydrophobic amino acids (Ala, Val, Leu, and Ile) and may suggest thermostability of protein. AI was higher for all BGLs analyzed in the present study suggesting their overall stability[108].

Comparative Analysis of Amino Acids Composition

ANOVA analysis demonstrated that Asp, Cys, Gln, His and Thr are significantly higher in M-BGLs than HT-BGLs and T-BGL homologs (P< 0.05, Table 5). These amino acids are unstable at higher temperature and undergo either oxidations

Table 5. Statistical analysis of amino acids composition (%) of GH1 β-glucosidases

Amino Acid	Average±SD			ANOVA analysis		Tukey multiple comparison, significant?		
	M-BGL	T-BGL	HT-BGL	F Value	P value	M vs T	M vs HT	T vs HT[a]
Ala (A)	9.02±2.5	8.56±2.7	7.28±2.2	2.605	0.083	No	No	No
Arg (R)	5.47±1.7	5.54±1.5	5.81±1.8	0.231	0.795	No	No	No
Asn (N)	4.23±1.1	3.98±1.1	4.70±1.4	1.792	0.176	No	No	No
Asp (D)	7.58±1.6	7.72±1.1	6.17±0.9	9.69	0.000	No	Yes	Yes
Cys (C)	0.95±0.7	0.66±0.4	0.40±0.4	5.463	0.007	No	Yes	No
Gln (Q)	3.18±1.1	2.38±1.0	1.86±0.7	10.439	0.000	Yes	Yes	No
Glu (E)	6.06±1.5	6.68±1.5	7.67±1.1	7.245	0.001	No	Yes	No
Gly (G)	8.2±1.3	8.47±0.8	7.65±0.9	3.54	0.035	No	No	Yes
His (H)	3.21±0.8	3.19±0.9	2.63±0.5	4.04	0.03	No	Yes	Yes
Ile (I)	5.2±1.5	5.39±1.8	5.8±1.6	0.723	0.49	No	No	No
Leu (L)	8.09±1.4	8.04±1.1	7.75±1.5	0.401	0.671	No	No	No
Lys (K)	3.71±1.8	4.76±2.7	5.84±2.1	4.54	0.015	No	Yes	No
Met (M)	1.81±0.7	1.89±0.7	2.1±0.8	0.859	0.429	No	No	No
Phe (F)	4.67±1.4	4.54±1.1	5.02±0.8	0.982	0.381	No	No	No
Pro (P)	4.89±1.1	4.89±1.2	5.21±1.2	0.537	0.586	No	No	No
Ser (S)	4.89±1.1	4.65±1.0	4.71±1.3	0.236	0.79	No	No	No
Thr (T)	5.29±0.8	4.48±1.1	3.48±0.7	19.996	0.000	Yes	Yes	Yes
Trp (W)	2.84±0.5	2.88±0.5	3.28±0.3	5.68	0.006	No	Yes	Yes
Tyr (Y)	4.93±0.8	5.5±0.9	6.08±0.5	11.554	0.000	No	Yes	Yes
Val (V)	5.84±1.2	5.81±1.3	6.63±1.2	2.9	0.063	No	No	No
nonpolar	43.03±3.7	42.57±3.2	42.40±3.1	0.186	0.831	No	No	No
polar	21.74±2.3	19.54±1.6	17.78±2.3	16.807	0.000	Yes	Yes	Yes
charged	22.81±2.2	24.86±2.3	25.48±1.8	8.058	0.001	Yes	Yes	No
aromatic	12.44±1.9	13.00±1.8	14.37±0.9	7.033	0.002	No	Yes	Yes

[a] M for M-BGL, T for T-BGL, and HT for HT-BGL.

or deamination at higher temperature explaining why they are less common in thermostable protein compared to mesophilic homologs[22,95,104,109-111]. Cys specifically plays a dual role by, on one hand, reducing thermostability through increasing internal cavities and oxidation at a higher temperature and, on the other, increasing thermostability through the formation of disulfide bonds which enhance protein rigidity and stability[112]. Conversely, Glu, Lys, Trp, and Tyr are significantly higher in HT-BGLs than their T-BGLs and M-BGLs counterparts (P<0.05, Table 5). Glu is negatively charged amino acids common in both exposed and buried region of the protein and involved in electrostatic interactions. Farias *et al.* (2003) found E+K increased and Q+H decreased in thermostable protein suggesting E+K/Q+H ratio can be used as an indicator of thermal stability[113]. Similarly, Lys is positively charged amino acid which involves in ionic interactions resulting in enhanced thermo-stability and hence it is more abundant in thermophilic and hyperthermophilic proteins[114-116]. Furthermore, both Trp and Tyr are aromatic amino acids which are more common in thermostable protein than their mesophilic homologs[13,117]. Aromatic amino acids contribute to protein thermostability through π-π and cation-π interactions[12,118]. Gly was significantly higher in T-BGLs than HT-BGLs or M-BGLs homologs (P<0.05, Table 5). Gly is small hydrophobic amino acid responsible for creating void or cavity in the interior of protein thus hyperthermostable protein are evolved to have less Gly content to minimize the cavities which may disturb protein upon temperature increase[104,117]. The analysis also showed that there is no significant difference in the means of nonpolar amino acids Ala, Ile, Leu, Met, Phe, Pro, Val, and polar amino acid Met, Arg, Asn, and Ser between M-BGL, T-BGL and HT-BGL homologs (P>0.05, Table 5). Ala is the best helix forming residue associated with increased thermostability and packing of the protein[119,120]. Ile was found to be more common in thermostable compared to mesophilic protein[100]. Phe is a hydrophobic amino acid that tends to bury inside protein thus was higher in hyperthermophilic protein than their meso- and thermophilic homologues[121]. Previous research reported that α-helices of thermophilic protein are more stable than those of mesophilic homologs perhaps due to the high abundance of amino acids with greater propensity to form α-helices (Ala, Leu, Arg) and low abundance in β-branch sheet forming residues (Val, Ile, Thr). α-helices of thermostable protein can also be stabilized by interactions between side chains of amino acids such as Glu and Arg[119,122,123]. Pro has pyrrolidine ring which allows it to have least conformational states and low conformational entropy restricting the configuration of preceding amino acids thus it is more common on rigid and turn conformations and hence reported to be higher in thermophilic protein[116]. Pro has been used to increase protein thermo-stability and can be considered, here, a potential hotspot to enhance thermostability of BGLs[124]. Similarly, Met, Asn, and Ser are thermo-labile that undergo either oxidation or deamination (Asn) at elevated temperature and are therefore less common in the thermostable protein[125,126]. Indeed, the substitution of Ser by Ala in thermophilic protein is widely reported[100]. Arg is a positively charged residue that participates in electrostatic bond formation to enhance protein stability[127,128]. The present study cannot justify why the residues such as Ala, Phe, Arg, and Pro which generally contribute to thermostability are not statistically higher in thermostable BGLs than mesophilic one. However, it is important to note that this study compared protein sequences solely from one family (GH1 BGLs) whereas previous studies compared protein sequences from several families; it is well-reported that different protein families adopt different strategies to enhance their thermostability[12].

Table 6. Multiple regression analysis of polar and aromatic amino acids for optimal temperature prediction

Variable	Coefficient	Std. Error	β	T value	Sig.
Intercept	31.018	25.984		1.194	0.238
Polar Amino Acids	-3.008	0.765	-0.374	-3.934	0.000
Tyr + Trp	10.687	1.942	0.523	5.504	0.000

Collectively, nonpolar amino acids (Ala, Gly, Ile, Leu, Met, Pro, Val) were the most abundant amino acids in all BGLs accounting for about 42.5% of total amino acids with no statistical difference in their means between M-, T-, and HT-BGLs (P>0.05, Table.5). Nonpolar amino acids are buried in the interior of protein and influence its hydrophobicity which is the major interacting force responsible for the stability of protein core[104,117]. Chakravarty *et al.* (2002) reported that nonpolar amino acids are relatively higher in thermophilic protein than their mesophilic protein[114]. Conversely, polar amino acids (Asn, Gln, Ser, Thr, His, Cys) are significantly higher in M-BGLs than T-BGLs and HT-BGLs (P<0.05, Table 5). Decrease of polar amino acids in thermostable enzymes contributes to thermostability by minimizing cavities, Gln- and Asn- induced deamidation, and Cys, Ser and Thr oxidation at higher temperatures. This finding is in agreement with previous reports[13,125,126,129]. In contrary, charged amino acids (Glu, Asp, Lys, Arg) are higher in HT-BGLs and T-BGLs than M-BGLs (P< 0.05, Table 5). Increase of charged amino acids in the thermostable protein was previously reported and appears to mediate protein thermostability through the formation of hydrogen and ionic interactions[115,126,130]. Finally, aromatic amino acids (Phe, Tyr, Trp) are also significantly higher in HT-BGL than M-BGL and T-BGL analogs (P<0.05). This increase in aromatic amino acids enhances thermostability by increasing hydrophobicity of protein through cation-π and π-π interaction[131] and compactness/packing of protein and decreasing cavities[106].

Multiple Regression Analysis

As previously demonstrated, the mean numbers of positively, polar, and aromatic amino acids are significantly different between M-, T-, and HT-BGLs with both positively and aromatic amino acids are directly correlated with optimal temperature (r= 0.62 and r= 0.65, respectively) and polar amino acids are negatively correlated (r= -0.55). These variables were used to perform multiple linear regression to determine a model for predicting optimal temperature. However, the positively charged amino acids were excluded from the model because it failed to be a significant predictor as indicated by individual test (P> 0.05). The model was constructed with polar and aromatic amino acids which significantly predicted optimal temperature with R square value of 0.53 indicating that variance in optimal temperature of 53% could be explained by the variation of these two groups of amino acids. This model also has multiple correlation coefficients R of 0.741 indicating that a high-quality prediction of this model. Additionally, β- coefficient indicates that aromatic amino acids (Trp+Tyr) contributed more to predicting optimal temperature than polar amino acids (Table 6). Of note, the low prediction value of this model (53%) is because thermostability cannot be solely predicted from the primary sequences of protein[132].

CONCLUSION

Thermostable BGLs differ from their mesophilic counterparts in several physicochemical properties such as molecular weight, isoelectric points, positively charged amino acids, and extinction coefficient. The high abundance of nonpolar amino acids in all BGLs may indicate general stability of BGLs. Additionally, increase in aromatic amino acids (Tyr and Trp) and decrease in polar amino acids (Gln, His, Thr, Cys) contributes significantly to BGL thermostability probably by combined mechanisms of increased hydrophobicity and decreases cavities of globular proteins. Charged amino acids (Lys and Glu) may also contribute to BGL thermostability through the formation of ionic bonds. Overall, these amino acids may be targeted through protein engineering for the conversion of mesophilic BGLs to their thermostable analogs. However, thermostability cannot be predicted solely from amino acids composition since the spatial arrangement of amino acids and structural feature of protein influence protein thermostability. Therefore, future analysis should focus on characterizing amino acids motifs and secondary structure of mesophilic and thermophilic BGLs to elucidate more attributes associated with thermostability. Furthermore, benefiting from a large number of X-ray crystallographic structures of BGLs elucidated to date, a comparative analysis of 3D structures may provide a deep insight into the difference between mesophilic and thermophilic BGLs thus paving the road toward successful protein engineering of this industrially valuable enzyme.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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None.

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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