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Research Article

In silico Analysis of Determinant Factors in Microbial Protease Thermostability

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Abstract

Thermostable proteases are one of the pivotal enzymatic groups which play fundamental roles in biotechnology-related industries. The identification of bacterial thermostable enzymes through screening programs is a time and cost consuming process. So, extensive bioinformatics and experimental studies have been conducted to reveal thermo stabilizing factors. The current study was aimed to evaluate distinctive indicators among 33 thermostable and 10 mesostable proteolytic enzymes. The frequency of individual amino acids, aliphatic indexes, melting temperatures, isoelectric points, as well as, the frequency of AXXXA and GXXXG motifs were determined and compared among these enzymes. In addition, types of proteolytic enzymes and their active sites were assigned. Moreover, the frequency of alpha helixes, polar surface regions, and packing volumes of these enzymes with the known structures were characterized. Results showed that the frequency of Ala and AXXXA motifs were significantly higher in thermostable proteolytic enzymes, while they possess lower contents of Met, His, Lys and Leu in comparison to mesostable enzymes (P<0.05). According to statistical analysis, thermostable proteolytic enzymes indicated meaningful lower packing volumes than mesostable enzymes (P<0.05). Findings of the current study in addition to more detailed investigations on the thermostability mechanisms of various protein families are essential for designing more efficient industrial enzymes with functional properties at high temperatures.

***Keywords*:** Bioinformatics analysis, Protein engineering, Proteolytic enzyme, Thermostability

Introduction

The application of biocatalysts in various industries is safer than using chemical compounds and has environmental advantages (Razzaq et al., 2019). Proteases, as one of the main industrial enzymes, are responsible for around 60% of the world enzyme market (Raveendran et al., 2018). Proteolytic enzymes constitute a very large and complex group of hydrolases. Despite the high diversity of proteases' functions and structures, they were simple classified to exo- or endo-proteases according to their site of cleavage (Souza et al., 2015). In addition, exo-peptidases can be further categorized into amino- and carboxy-peptidases (Souza et al., 2015). Proteolytic enzymes also can be grouped based on residues in their catalytic active sites into serine proteases (Patel, 2017), aspartic proteases, asparagine proteases, cysteine proteases (Dadshahi et al., 2016), metalloproteases (Abebe et al., 2014), glutamic proteases, threonine proteases or proteases with mixed or unknown catalytic mechanisms (Rawlings et al., 2007; Rawlings et al., 2017).

Proteases are widely produced by all organisms, including plants, animals, fungi, bacteria, and archaea. Microbial-derived proteases have been applied for commercial purposes due to easier large-scale production (Wang et al., 2008; Haddar et al., 2010). Microbial proteases are deemed vital elements in a wide range of processes including nutritional, pharmaceutical, environmental, detergent, textile, leather, and livestock industries (Homaei et al., 2010; Homaei and Etemadipour, 2015; Barzkar et al., 2018). However, the primary limitation to the application of microbial proteases is their instability under high temperature and pH conditions (Iqbalsyah, et al., 2019). Hence, thermostable and/or thermophilic proteases which possess high abilities to preserve their activities under harsh conditions of industrial processes are more applicable in the field of biotechnology (Wakarchuk et al., 1994).

Thermostable and thermophilic enzymes are normally derived from thermophilic and mesophilic organisms. Most of the thermostable and/or thermophilic proteases are not only stable at high temperatures but also preserve their catalytic activities in the presence of detergents and other

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denaturing chemicals, such as urea, guanidine-HCl, Dithiothreitol, 2-mercaptoethanol, and chaotropic agents. The application of thermostable and/or thermophilic proteases in the industrial processes have some advantages, including higher reaction rates, enhanced substrate solubility, and decreased solution viscosities. In addition, their large scale production is less susceptible to contamination since there are fewer microorganisms which can grow at high temperatures. However, screening of thermophilic microorganisms for finding thermophilic proteases is a tedious, costly, and time-consuming strategy. In this regard, engineering mesostable enzymes to develop thermostable enzymes is considered as a valuable strategy (Kumar, 2002; Li and Li, 2009: Tavano et al., 2018).

To determine factors which are responsible for thermal stability of proteins several approaches can be applied. Among which, we mention *i)* bioinformatic comparison of the protein structure for thermophilic proteins versus their mesophilic homologues, *ii)* computational studies on a dataset of thermophilic and mesophilic proteins to compare their various features, *iii)* mutational-based studies, and *iv)* comparing whole genome sequences of thermophilic species with their mesophilic homologues (Sadeghi et al., 2006).

In point of structural view, thermostable proteins have higher numbers of ionic interactions, salt bridges, disulfide, and hydrogen bonds (Sadeghi et al., 2006). They have increased packing density (Sadeghi et al., 2006), higher contents of helical structures (Sadeghi et al., 2006), less short surface loops (Sadeghi et al., 2006), decreased surface area to volume ratios (Das and Gerstein, 2000; Tekaia et al., 2002), and decreased internal cavities (Pellegrini et al., 1999). At the level of amino acid sequences, thermophilic proteins have a higher frequency of hydrophobic (Razvi and Scholtz, 2006) and charged amino acids, higher relative content of Arg, Glu (Pack et al., 2013), lower occurrence of bulky polar residues, decreased contents of uncharged polar residues (Ser, Thr, Asn, and Gln) (Haney et al., 1999), and increased contents of aromatic residues (Chakravorty et al., 2011).

Therefore, further investigations about the thermostability encountered mechanisms are essential for theoretical description of protein folding and stability and also, for designing efficient thermostable industrial enzymes. Since thermostable microbial proteases have a key contributory role in the market of industrial enzyme, and whereas there are no shared particular sequences or structural patterns among heat-stable proteases, focusing on this group of enzymes is worthwhile. Here, we selected 43 thermostable/thermophilic and mesophilic proteases which their characteristics had been previously determined in experimental studies. This study, innovatively investigated the consistency of well-known thermostability parameters among thermostable proteolytic enzymes.

Materials and Methods

Sequence collection of microbial proteases

Thirty-three bacterial thermostable proteolytic enzymes, which their optimum temperature and/or thermal stability had been reported in experimental studies were selected from UniProt proteomic server (Consortium 2018). Moreover, ten well-identified mesostable proteolytic enzymes' sequences were also collected from UniProt to compare with thermostable ones. In this regard, amino acid sequences of these enzymes were retrieved in FASTA format.

Investigation the thermostability properties of different proteases

The amino acid composition, aliphatic index, and isoelectric point of each proteolytic enzyme were determined through the ProtParam tool (web.expasy.org/protparam/). Melting temperatures (Tm) and net charges of the enzymes at pH 7 were calculated using Tm Predictor (tm.life.nthu.edu.tw/) and PepCalc (pepcalc.com), respectively. Frequency of AXXXA and GXXXG motifs in the sequences were assigned through manual searching and counting.

Determination of the types of proteolytic enzymes and their active sites

The types of the proteases were determined based on their conserved domain composition using CD-search tool (Marchler-Bauer et al., 2016). The active sites of proteolytic enzymes with protease activity were identified through the literature review (Yamagata and Ichishima, 1995; Wu et al., 2004; Pombejra et al., 2018). To determine the active sites of enzymes with peptidase activity, the MEROPS database (Rawlings et al., 2017) in addition to literature review (Medrano et al., 1998; Goldstein et al., 2005; Bjelke et al., 2006; Ohara-Nemoto et al., 2014; Reddi et al., 2014) were applied.

Multiple sequence alignments

In order to determine the possible differences of conserved motifs and residues in the sequences of mesostable and thermostable proteases, multiple

sequence alignments were performed in IBIVU server using PRALINE software (Simossis and Heringa, 2005). Since the investigated amino acid sequences have significantly different lengths (285-1364 amino acid residues), matrix PAM 250 with gap opening 10 and gap extension 1 were used for alignments.

Structural analyses

Among 43 selected proteolytic enzymes, nine enzymes had a tertiary structure in the Protein Data Bank (PDB) (Bank, 2000) which four and five of them are mesostable and thermostable/thermophilic proteolytic enzymes, respectively. Structural analysis of these enzymes was performed using VADAR (Volume, Area, Dihedral Angle Reporter) software version 1.8 which can be accessed at http://redpoll. pharmacy.ualberta.ca/vadar/ (Willard et al., 2003). Furthermore, frequency of alpha helixes, polar surface regions, and packing ratios in these structures were determined. One of these structures (PDB ID: 5J44, which was related to Q8VSL2 sequence) with more than 2000 residues, was not investigable using VADAR software.

Statistical analyses

Homogeneity of variance and normal distribution of data including frequency of each twenty amino acids, AXXXA and GXXXG motifs, the mean of pI (isoelectric point), aliphatic indexes, Tm, net charges in pH 7, melting temperatures in both thermophilic/thermostable and mesostable enzymes groups were evaluated using Levene and Shapiro Wilk tests in SPSS version 23, respectively. To compare the mean of each variable in two investigated subsets (thermophilic/thermostable and mesostable proteolytic enzymes), independent *t*-test and Mann-Whitney test were applied for data with or without normal distribution, respectively. Finally, chi-square test was carried out to evaluate the possible differences in melting temperatures of proteolytic enzymes in these two groups.

Results

Source and properties of investigated sequences

All selected enzymes have bacterial origin except Q2QC89, which has been derived from an archaeon (*Thermococcus* sp.).

Proteolytic enzymes in the current study with an optimum temperature of 50°C or higher and/or thermal stability in the mentioned temperatures were considered as thermostable enzymes, and the others were placed in the mesostable group. Some properties of the proteolytic enzymes which were obtained from literature are summarized in table 1.

Distinctive features of thermophilic/thermostable proteolytic enzymes

Some characteristics of the enzymes which are related to the thermostability were summarized in table 2. The Tm Predictor categorizes proteins based on their melting temperatures in three ranges; >65°C, <55°C and 55-65°C. Statistical analysis showed that there were no significant differences in melting temperatures of thermophilic/thermostable and mesophilic proteolytic enzymes ($P>0.05$).

In addition, statistical analysis confirmed normal distributions of data related to frequency of AXXXA motifs, and amino acids except Ala, Arg, Cys, Gln, His, Leu, Phe ($P>0.05$), while isoelectric points, net charges, aliphatic indexes, and GXXXG motifs did not show normal distributions ($P<0.05$). In case of pI, aliphatic index and net charge parameters, thermostable and mesostable enzymes were similar; however, the frequency of AXXXA motif was significantly higher in thermophilic/thermostable proteolytic enzymes when compared to mesostable ones ($P<0.05$).

The percentages of amino acids present in proteolytic enzymes are presented in Figure 1. According to the *t*-test and Mann Whitney results, there was a significant difference in frequencies of Met, Ala, Leu, Lys and His between thermostable proteolytic enzymes and mesostable ones. Although, thermophilic/thermostable enzymes had higher percentages of Ala (9.2% *vs.* 8.59% in the other group) and lower percentages of Met (2.01%), Leu (7.16%), Lys (5.06%) and His (2.32%) in comparison to mesostable proteins. The frequency of the above-mentioned residues in the mesostable enzymes were as follows: Met (2.68%), Leu (8.22%), Lys (5.84%), and His (2.61%) (Figure 1). Moreover, to compare each of the twenty amino acids, the analysis of the hydrophobic, aromatic, polar charged, and polar uncharged amino acid groups was performed; but, the subsets of the residues did not show any significant differences between thermostable and mesostable enzymes.

Types of proteolytic enzymes and their active sites

Whereas the types of some proteolytic enzymes were not precisely determined in the previous studies, the retrieved sequences were classified according to their conserved domains. It was confirmed that 25 and 18 of investigated enzymes could be grouped as proteases and peptidases,

respectively (Table 2). Two types of catalytic triads within conserved sequences were observed among the proteases, which are including Asp, His, and Ser or His, His, and Glu. However, peptidases due to their variability had different active sites, and one of them (UniProt ID: G5DCB7) belongs to the peptidase family with unknown catalytic mechanism based on the MEROPS database (Table 2).

Table 1. Investigated microbial proteolytic enzymes and some of their properties obtained from literature.

UniProt ID	Source of microorganisms	Optimum temperature	Thermal stability	Optimum pH	pH stability range	References
Thermostable/thermophilic enzymes						
P06874	*Bacillus stearothermophilus*	*ND*	65ºC	7	*ND*	(Fujii et al. 1983)
P43133	*Bacillus stearothermophilus*	*ND*	65ºC	*ND*	6.5-7.5	(Kubo and Imanaka 1988)
P23341	*Thermus aquaticus* YT-1	75-80ºC	*ND*	*ND*	*ND*	(Motoshima et al. 1990)
P39899	*Bacillus subtilis*	ND	65ºC	6.6	*ND*	(Tran et al. 1991)
P23384	*Bacillus caldolyticus*	77ºC	*ND*	7	*ND*	(Van den Burg et al. 1991)
P42663	*Thermus aquaticus* YT-l	80ºC	*ND*	8	*ND*	(S.-H. Lee et al. 1992)
P41363	*Bacillus* sp. no. AH-101	*ND*	30-70ºC	ND	12-13	(Takami et al. 1992)
P0CH29	*Bacillus megaterium* ATCC 1458 1	58ºC	*ND*	6.4-7.2	*ND*	(KÜHN and FORTNAGEL 1993)
Q45670	*Bacillus* sp. strain AK.1	75ºC	*ND*	8.5	*ND*	(Maciver et al. 1994)
Q45621	*Bacillus* sp. NKS-21	50ºC	*ND*	*ND*	6-11	(Yamagata and Ichishima 1995)
P80146	*Thermus* sp. strain Rt41A	*ND*	70ºC	8	*ND*	(Munro et al. 1995)
P04189	*Bacillus subtilis*	*ND*	50ºC	8	*ND*	(Kamal et al. 1995)
Q43880	*Bacillus sp.*	82ºC	*ND*	*ND*	*ND*	(Vecerek and Kyslik 1995)
Q99405	*Bacillus* sp. KSM-K16	55ºC	*ND*	10	*ND*	(Kobayashi et al. 1995)
Q56365	*Thermoactinomyces* sp. E79	85ºC	*ND*	11	5-12	(J.-K. Lee et al. 1996)
Q59223	*Bacillus* sp. strain EAI	*ND*	85-95ºC	6.5	6-7.5	(Saul et al. 1996)
P74937	*Thermoactinomyces* sp. HS682	65ºC	*ND*	11	6-12	(Tsuchiya et al. 1997)
O33599	*Staphylococcus aureus*	*ND*	100ºC	*ND*	5-8	(Ramadurai et al. 1999)

Q93JY4	*Prevotella albensis* M384	*ND*	60ºC	*ND*	7-8	(Walker et al. 2003)
Q6W4N2	*Bacillus* sp. WF146	58ºC	*ND*	8	*ND*	(Wu et al. 2004)
Q84FM9	*Fervidobacterium islandicum*	80ºC	*ND*	8	*ND*	(Gödde et al. 2005)
Q2QC89	*Thermococcus* sp.NA1	*ND*	70-80ºC	6.5	*ND*	(H. S. Lee et al. 2006)
Q8G6Z9	*Bifidobacterium longum*	50ºC	40-60ºC	8	4-8	(Seo et al. 2007)
E0XH65	*Bacillus* sp. B001	60ºC	20-90ºC	10	5-12	(Deng et al. 2010)
G8HV17	*Bacillus circulans* MTCC 7906	60ºC	*ND*	9	*ND*	(Kaur et al. 2012)
G5DCB7	*Geobacillus thermoleovorans* DSM 15325	50ºC	*ND*	7.4	*ND*	(Jasilionis et al. 2012)
J9XWB6	*Serratia* sp. ZF03	*ND*	50-55ºC	8	8-10	(Salarizadeh et al. 2014)
H2BKX5	*Myroides profundi* D25	60ºC	*ND*	8.5	*ND*	(Ran et al. 2014)
W5RWH8	*Geobacillus thermoleovorans* DSM 15325	40ºC	50-60ºC	7.3	5-8	(Jasilionis and Kuisiene 2015)
A0A0C4XY83	*Streptomyces* sp.M30	75ºC	*ND*	9	6-11	(Xin et al. 2015)
H6WCS0	*Dichelobacter nodosus*	35ºC	15–65ºC	7	4-10	(Wani et al. 2016)
Q3HTI0	*Bacillus cereus* PMW8	60ºC	40-70ºC	9	*ND*	(Esakkiraj et al. 2016)
Q45300	*Bacillus licheniformis*	60ºC	*ND*	10	*ND*	(Ramakrishnan et al. 2017)
Mesostable enzymes						
P46544	*Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397	37ºC	*ND*	*ND*	*ND*	(Atlan et al. 1994)
P94870	*Lactobacillus helveticus* CNRZ32	*ND*	32-37ºC	4.5	*ND*	(Fenster et al. 1997)
O07121	*Lactococcus lactis* MG1363	37ºC	*ND*	*ND*	*ND*	(Hellendoorn et al. 1997)
Q8VSL2	*Shigella flexneri*	37ºC	*ND*	7.5	*ND*	(Benjelloun-Touimi et al. 1998)
Q7MUW6	*Porphyromonas gingivalis*	*ND*	25-37ºC	ND	6-8	(Banbula et al. 1999)
Q9L4G1	*Lactobacillus helveticus*	*ND*	25-37ºC	7.5	6-8	(Savijoki and Palva 2000)
O82882	*Escherichia coli* O157:H7	37-42ºC	*ND*	6.5-7	*ND*	(Grys et al. 2006)
Q29ZA8	*Bacillus intermedius*	37ºC	*ND*	8	*ND*	(Sharipova et al. 2008)

P9WK19	*Mycobacterium tuberculosis* H37Rv	37ºC	*ND*	7.5	*ND*	(Zhang et al. 2009)
B2RIT0	*Porphyromonas gingivalis*	37ºC	*ND*	6	*ND*	(Ohara-Nemoto et al. 2014)

ND: Not Determined

Table 2. *In silico* characterization of investigated proteolytic enzymes.

UniProt ID	Types of proteases	Active sites	pI	Aliphatic Index	Tm	Net charge in pH	Number of AXXXA motifs	Number of GXXXG motifs
P06874	Zn-dependent metalloprotease	H374, H378, E398	5.68	76.72	<55	-8.9	8	2
P43133	Zn-dependent metalloprotease	H377, H381, E401	5.82	76.46	55-65	-6.9	6	3
P23341	Aminopeptidase T	Y352	5.31	89.49	>65	-13.9	7	2
P39899	Zn-dependent metalloprotease	H369, H373, E393	5.43	73.10	55-65	-17.3	6	4
P23384	Zn-dependent metalloprotease	H370, H374, E394	5.64	75.33	<55	-8.2	8	4
P42663	Carboxypeptidase	E277	5.53	83.76	55-65	-13.6	6	6
P41363	Serine protease	D124, H154, S307	6.56	90.80	>65	-1	4	1
P0CH29	Zn-dependent metalloprotease	H388, H392, E412	8.39	70.64	55-65	4	6	5
Q45670	Thermitase-like proteins	D160, H193, S347	4.68	82.97	>65	-17.5	7	1
Q45621	Serine protease	D49, H86, S250	4.83	90.50	55-65	-17.5	3	1
P80146	Proteinase K-like proteins	D171, H204, S356	6.16	90.98	>65	-2.6	5	2
P04189	Serine protease	D138, H170, S327	9.04	81.23	>65	5.8	5	3

Q43880	Zn-dependent metalloprotease	H372, H376, E396	5.47	73.97	<55	-10.1	7	3
Q99405	Serine protease	D143, H173, S326	4.67	91.18	>65	-19.2	5	1
Q56365	Serine protease	D143, H176, S330	6.04	78.57	>65	-3.3	8	1
Q59223	Thermitase-like proteins	H372, H376, E396	5.39	74.51	55-65	-11.1	7	3
P74937	Zn-dependent metalloprotease	D49, H86, S249	4.92	87.13	>65	-15.6	3	0
O33599	Metallopeptidase	H291	6.16	38.10	55-65	-4.6	2	2
Q93JY4	Dipeptidyl peptidase IV	Y511, S600, D674, H706	7.56	70.77	55-65	2.1	5	2
Q6W4N2	Serine protease	D149, H185, S363	5.28	81.41	>65	-8.5	7	3
Q84FM9	Serine protease	D210, H248, S424	5.45	93.84	>65	-9.6	3	4
Q2QC89	Zn-dependent carboxypeptidase	E297	5.61	84.97	>65	-11.7	2	2
Q8G6Z9	Dipeptidase	C3	4.46	71.07	55-65	-47	2	0
E0XH65	Serine protease	D138, H168, S321	4.0	84.08	>65	-40.5	3	1
G8HV17	Serine protease	D155, H187, S384	5.14	79.41	55-65	-14.7	3	2
G5DCB7	Collagenase-like protease	Unknown	5.39	88.06	>65	-13.4	4	1
J9XWB6	Serralysin	E175	4.63	64.18	>65	-23.8	3	4
H2BKX5	Kp43 proteases	D129, H177, S378	5.85	79.68	55-65	-8	7	2
W5RWH8	Oligo peptidase F	E401	8.90	87.28	>65	9.2	9	3
A0A0C4XY83	Proteinase K-like proteins	D156, H187, S339	4.25	80.33	>65	-21.4	7	3
H6WCS0	Serine protease	D156, H220, S392	6.25	78.92	55-65	-4	5	3

Q3HTI0	Zn-dependent metalloprotease	H143, H147, E167	5.18	65.65	55-65	-8.3	6	2
Q45300	Serine protease	D137, H168, S325	8.94	84.70	>65	4.8	4	2
P46544	Proline iminopeptidase	S107, D246, D273	5.09	87.69	>65	-12.4	3	2
P94870	Aminopeptidases	Q64, C70, H362, N383	5.21	75.46	>65	-13.2	1	2
O07121	Dipeptidase	H92	4.73	79.87	>65	-33.7	4	3
Q8VSL2	Serine peptidase	H134, D162, S267	6.10	74.02	>65	-8.9	7	14
Q7MUW6	Dipeptidyl peptidase IV	Y518, S603, D678, H710	6.16	74.44	55-65	-8.2	4	6
Q9L4G1	Tripeptidase	D84, E178	4.77	73.90	>65	-29.8	1	2
O82882	Metallopeptidases	E447	6.39	74.59	55-65	-5.8	5	8
Q29ZA8	Serine protease	D138, H170, S327	8.96	80.73	>65	4.8	5	1
P9WK19	Methionine aminopeptidase	C105	5/07	87.89	55-65	-11.5	3	2
B2RIT0	Dipeptidyl aminopeptidase	S542, D627, H659	5.75	68.52	55-65	-10.7	4	4
Significant value			*NS*	*NS*	*NS*	*NS*	**	*NS*

NS, * and ** represent no significant differences, significant differences at 90% and 95% confidence intervals, respectively.

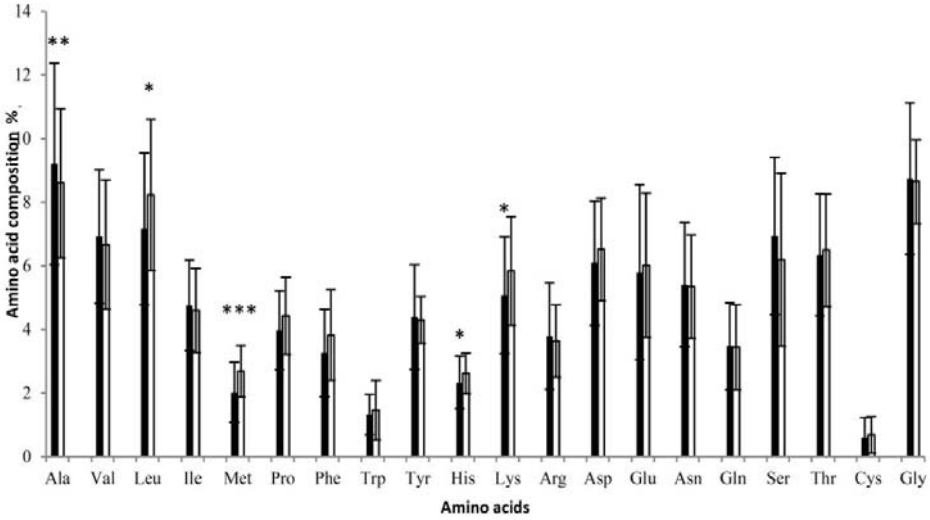

Figure 1. Frequency of different amino acids is reported for thermostable (black columns) and mesostable (white columns) groups. *, ** and *** represent significant differences at 90%, 95%, and 99% confidence intervals, respectively.

Sequence alignments

Results obtained through multiple sequence alignments indicated somewhat conserved sequences (from amino acid residues 1000 to1300) which are shown in supplementary file 1.

The structural features of thermostable/ proteolytic and mesostable enzymes

The numbers of alpha helixes, polar surface regions, and total volumes of available structures were predicted (Table 3). The statistical analyses showed that among structural characteristics, just total volumes of thermophilic/thermostable proteolytic enzymes were significantly lower than that of mesostable ones (P<0.05).

Discussion

In recent years, considerable efforts have been made to understand influential factors involved in thermal stability of thermostable or thermophilic proteins derived from mesophilic, thermophilic or

Table 3. Structural analyses of thermophilic/thermostable proteolytic enzymes and mesostable ones using VADAR software.

UniProt ID/ PDB ID	Type of enzymes	Alpha helix (%)	Polar surface Exposed)regions (polar ASA[1] (A^o) 10^{-2}	Total volume (Packing) 10^{-3} (A^o)
P43133/5WR3	**Thermophile/Thermostable**	37	4374.7	40568.7
Q45670/1DBI		28	3188.9	33870.1
P04189/1SCJ		96	3713.3	42181.7
Q99405/1MPT		78	3197.2	31663.4
O33599/1QWY		23	3810.9	29772.0
Q7MUW6/2D5L	**Mesostable**	96	6132.5	92421.4
O82882/3UJZ		56	6581.3	82474.0
P9WK19/1Y1N		71	2086.1	36599.7
Significant value		*NS*	*NS*	**

NS, and ** represent no significant difference and significant difference at 95% confidence interval, respectively.
[1] Accessible surface area.

hyper-thermophilic organisms. Thermophilic proteins preserve their native structures and consequently their activities under harsh environmental conditions, while, their mesostable homologs already denature. Comprehensive knowledge about factors involved in thermostability can be applied as a promising approach in developing thermostable enzymes through protein engineering. Most of the studies which have been worked on the thermal stability of proteins did not generally focus on a family or a limited group of proteins (Kumar et al., 2000; Liang et al., 2005; Sadeghi et al., 2006; Gromiha and Suresh, 2008). It seems that it is a reason for inconsistent results.

One of the fundamental features which profoundly affected structural, functional and biological properties of proteins is their amino acid composition. Analysis of amino acid composition and frequency can provide beneficial data about the importance of each amino acid in thermal stability of proteins. This feature can be different from one protein to another one in each organism or it can be taxon-specific (Zhou et al., 2008).

The current study showed that thermophilic or thermostable enzymes possess a higher frequency of Ala compared to mesostable ones. This residue is an appropriate helix former (Chakravorty et al., 2011) and through hydrophobic interactions provides conformational stability in the inner parts of proteins (Creighton, 1993). This feature leads to better packing, higher rigidity, hydrophobicity, and consequently more thermostability of proteins (Chakravarty and Varadarajan, 2000). This finding is analogous with previously published studies (Argos et al., 1979; Chakravarty and Varadarajan, 2000; Pack and Yoo, 2004). The percentage of Leu, another nonpolar residue, was lower in thermostable proteases than the mesostable ones. In case of Leu reported frequencies are not similar in various studies. Taylor and Vaisman detected higher percentages of Leu in thermophilic proteins (Taylor and Vaisman, 2010); while, Chakravarty and Varadaragan observed an opposite trend (Chakravarty and Varadarajan, 2000).

The present study indicated that thermophilic or thermostable proteases possess lower frequencies of thermolabile Met residue. This finding is in consistent with a previous finding (Kumar et al., 2000; Xu et al., 2003). The frequency of the other thermolabile amino acids, including Asn, Gln, and free Cys, in spite of their deamination or oxidation at high temperatures (Tomazic and Klibanov, 1988; Russell et al., 1994; Catanzano et al., 1997; Kumar et al., 2000; Xu et al., 2003), did not show indictable differences among the sequences. In the research of Kumar et al., among these temperature sensitive residues, only Cys was significantly lower in thermophilic proteins (Kumar et al., 2000).

Polar charged amino acids, including Arg, Lys, His, Asp, and Glu, contribute to the electrostatic interactions and enhancement of thermostability (Dill, 1990; Creighton, 1993; Ladbury et al., 1995; Vogt et al., 1997; Kumar et al., 2000). Although, in the current study, no significant differences were observed in the frequencies of Arg, Asp, and Glu between thermostable and mesostable proteases. Furthermore, lesser contents of Lys and His observed in thermostable proteins compared to mesostable ones. There are inconsistent reports about Lys frequency. Taylor and Vaisman found the lower percentages of Lys in thermophilic proteins (Taylor and Vaisman, 2010), while Cambillau and Claverie reported a higher percentages of Lys in hyperthermophiles (Cambillau and Claverie, 2000). Lower frequency of His in thermophilic proteins also reported in previous studies (Chakravarty and Varadarajan, 2000; Pack and Yoo, 2004; Sadeghi et al., 2006).

The aliphatic index, which is calculated based on the presence of amino acids with aliphatic side chains (Zhou et al., 2008), and melting temperature (Kumar et al., 2000) are two parameters which have positive effects on protein thermostability. However, in the present comparison, no significant differences were found between mesostable and thermostable groups for these criteria. Panja et al. showed that thermophilic proteins had a negative net charge at neutral pH and a slight acidic pI (Panja et al., 2015); while, thermostable and thermophilic proteolytic enzymes did not show any differences at the significant level of 0.05 in their study. Therefore, it seems that these factors are not determining agents for thermostability of proteases.

The AXXXA motif through helical interactions creates more stability in protein structures (Kleiger et al., 2002; Chakravorty et al., 2011). Higher frequency of AXXXA motif and poly Ala residues were also revealed through sequence analysis of the thermostable *Bacillus* lipases (Chakravorty et al., 2011). However, thermostable sequences in this study in spite of having significant higher Ala residues and AXXXA motifs did not have a higher percentages of alpha helixes in comparison with mesostable ones. If more PDB structures were available for the studied proteases, these contradictory findings might not occur. Since, limited structures of selected proteases and peptidases were available, the comprehensive

structural analyses were not possible. The exposed polar surfaces have been proposed as an effective factor for thermo stabilization of lipases (Chakravorty et al., 2011); however, our results were opposite to this expectation. Only packing volumes of the structures were significantly higher in thermostable enzymes than mesostable ones.

Investigating the thermostable and mesostable sequences through multiple sequence alignments is beneficial for finding short consensus sequences to design degenerate primers (Morya et al., 2012). Furthermore, these alignments will provide better insights for substitution of amino acids in protein engineering studies. Here, no considerable distinction was observed among the active sites or consensus motifs of thermostable and meostable enzymes.

Conclusion

In conclusion, according to the present study, thermostability of proteolytic enzymes not only can be attributed to the higher percentages of Ala, and fewer frequencies of His, Lys and Met, but also can be due to the presence of higher contents of AXXXA motifs and more packing structures in comparison to mesostable proteases. Without a doubt, the results of comparative studies between thermophilic proteins and their mesostable counterparts will revealed some thermo stabilizing factors. However, these results could not be accepted as general modes governing protein thermostability, because they completely depend on studied protein families. In addition, it is so difficult to relate any single factor as the main reason which is responsible for enhancement of thermal stability (Szilágyi and Závodszky, 2000). Therefore, extensive bioinformatic-based studies should be carried out on various thermostable and mesostable protein groups. Furthermore, the contradictory results should be taken into account to reach a reliable data and to enhance the thermal stability of mesostable proteins with desirable properties during protein designing or re-engineering processes.

Conflicts of interest

The authors declare that they have no conflict of interest.

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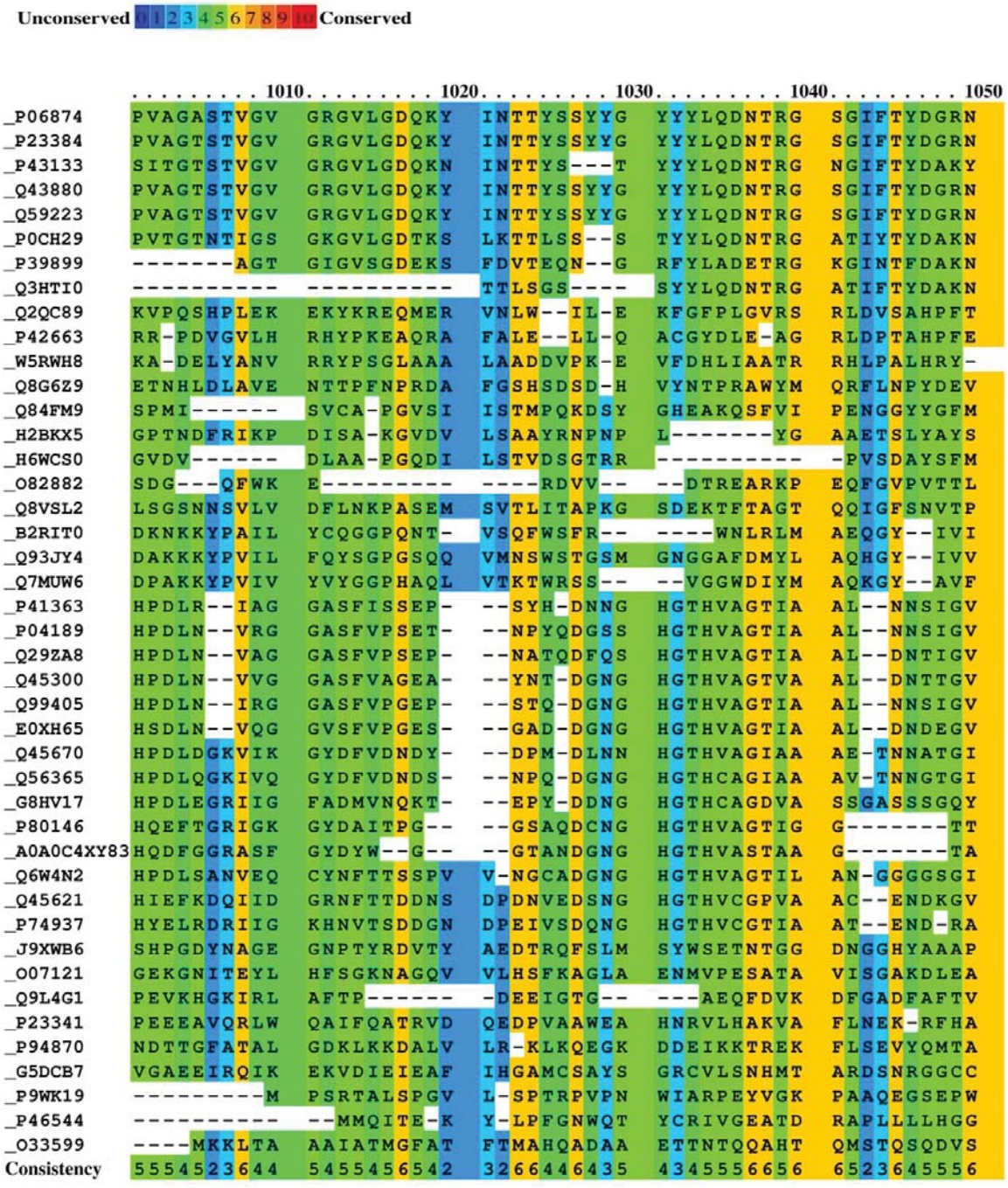
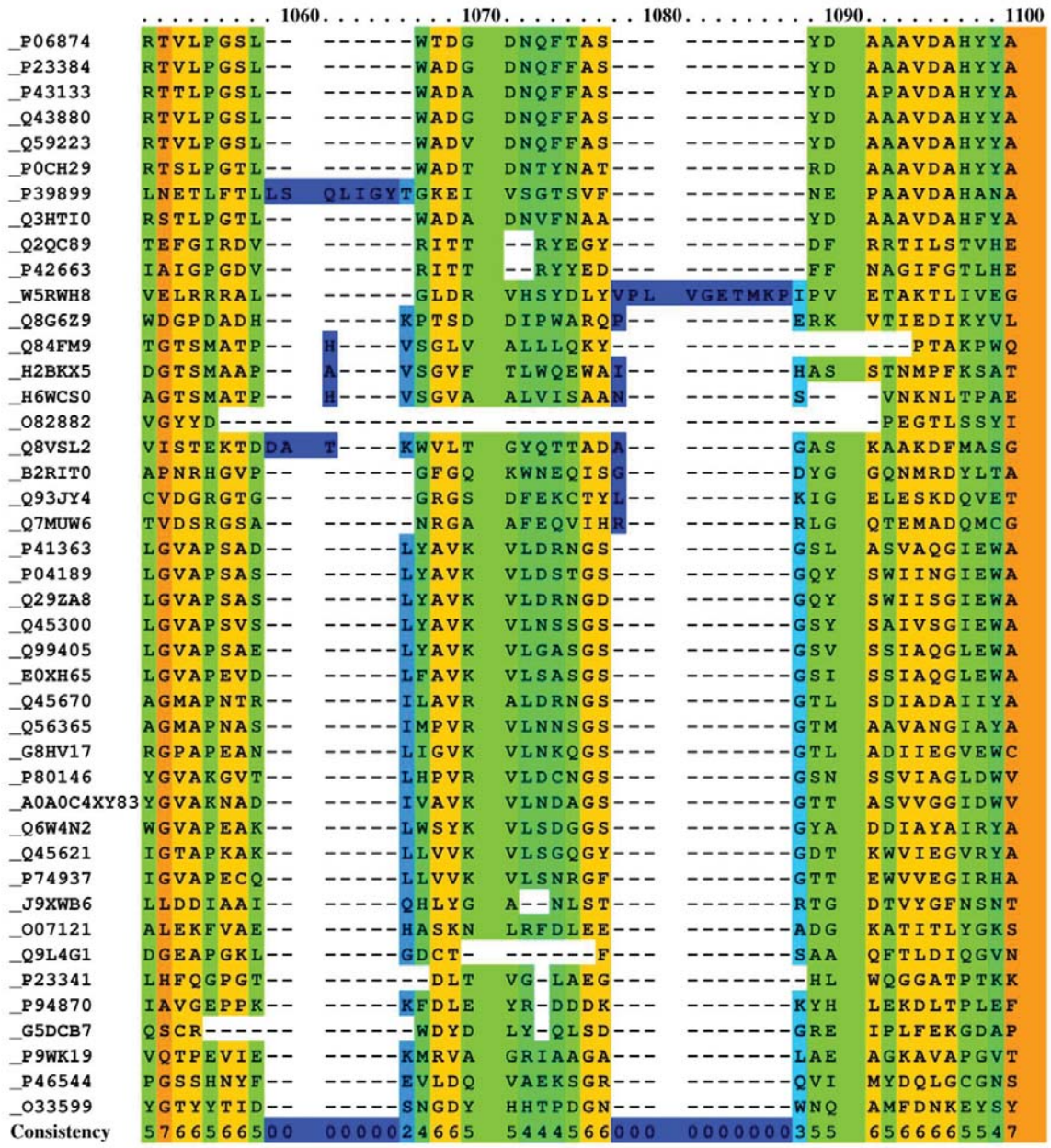
Supplementary Materials:

Supplementary file 1

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_P42663
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_Q8G6Z9
_Q84FM9
_H2BKX5
_H6WCS0
_O82882
_Q8VSL2
_B2RIT0
_Q93JY4
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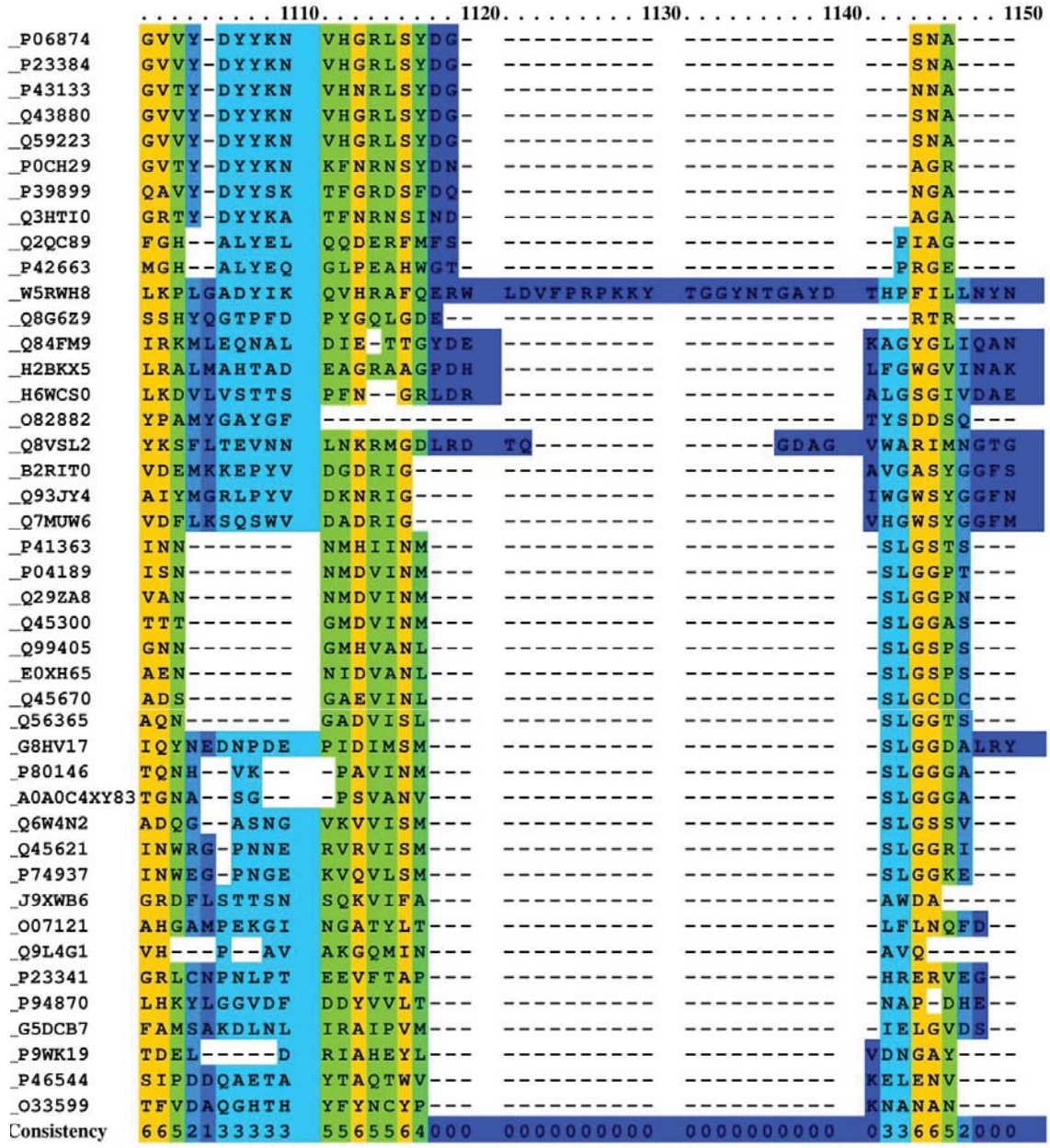
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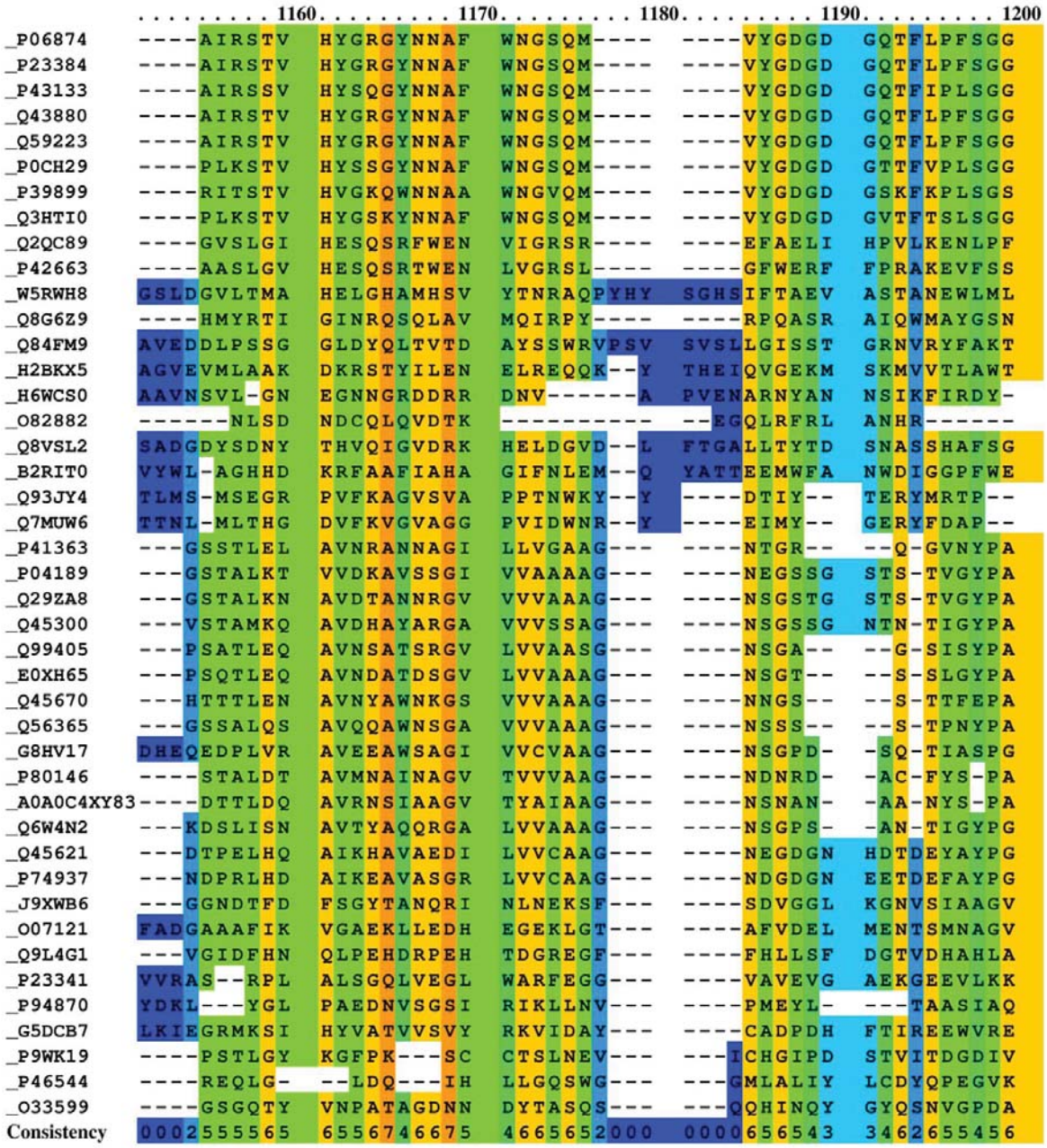
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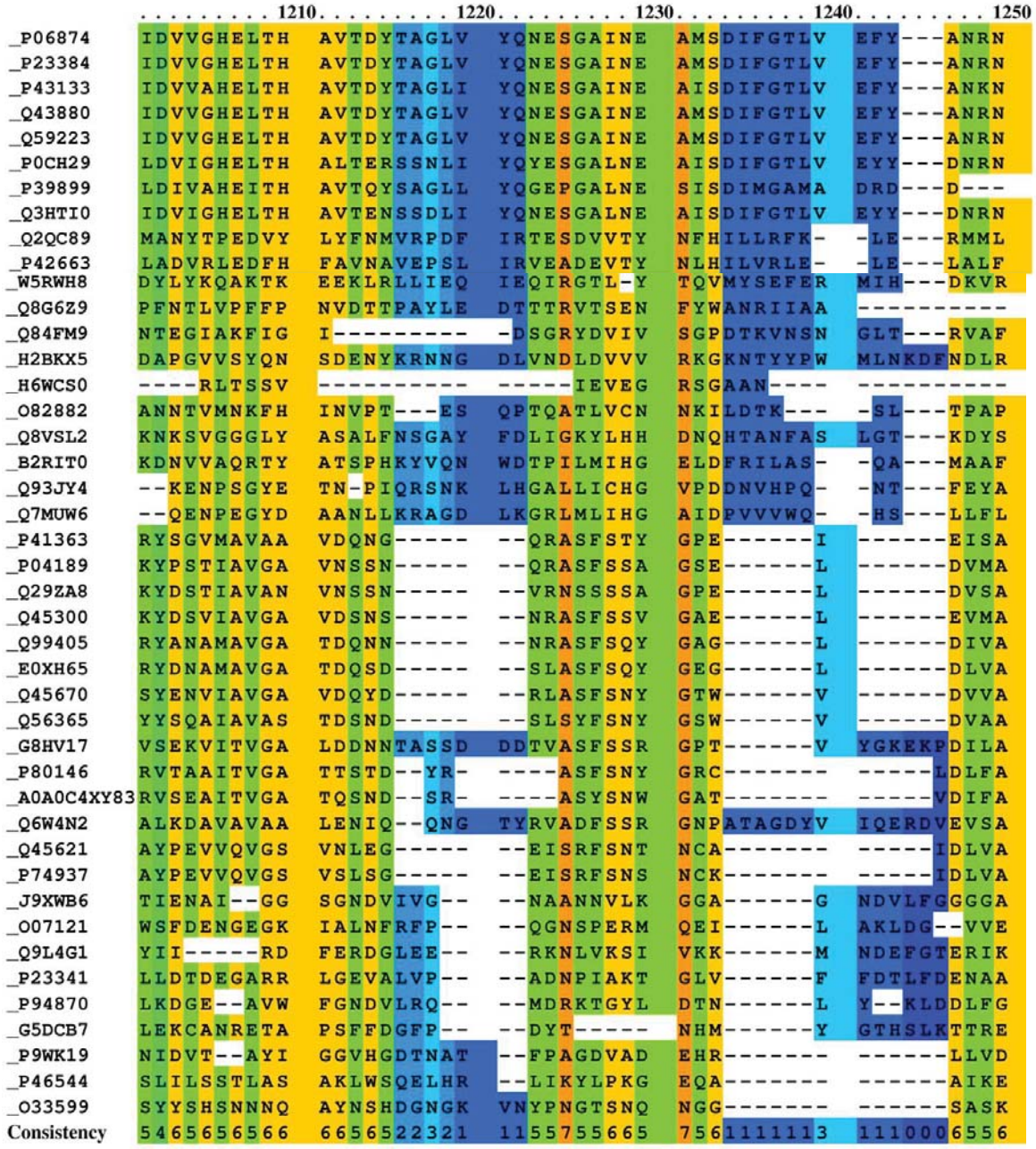
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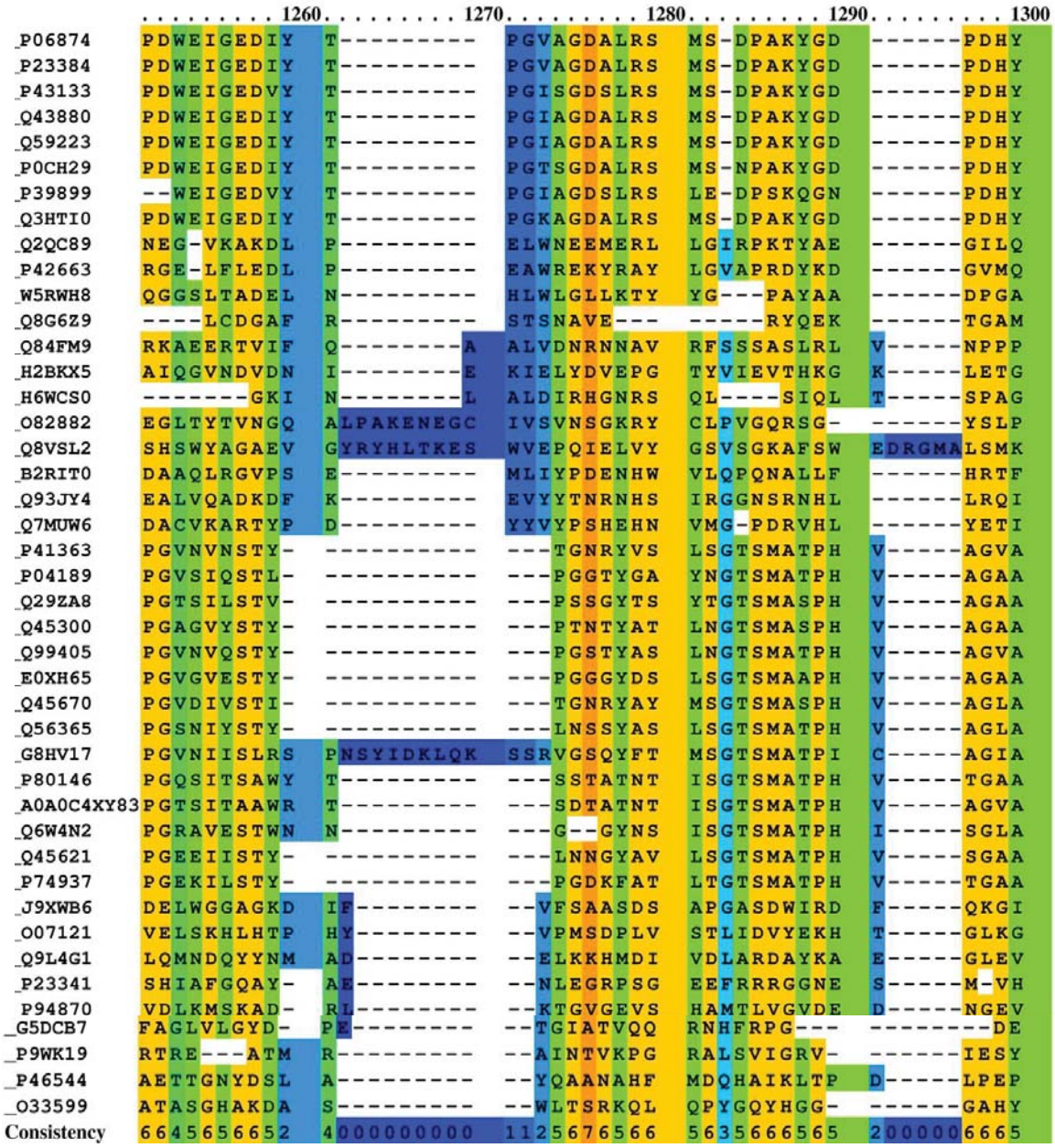

Multiple sequence alignment of the thermostable proteolytic enzymes. More conserved parts of the alignment have been presented from amino acid residues from 1000 to 1300