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Surface charge engineering of a *Bacillus gibsonii* subtilisin protease

Felix Jakob · Ronny Martinez · John Mandawe · Hendrik Hellmuth · Petra Siegert · Karl-Heinz Maurer · Ulrich Schwaneberg

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Abstract In proteins, a posttranslational deamidation process converts asparagine (Asn) and glutamine (Gln) residues into negatively charged aspartic (Asp) and glutamic acid (Glu), respectively. This process changes the protein net charge affecting enzyme activity, pH optimum, and stability. Understanding the principles which affect these enzyme properties would be valuable for protein engineering in general. In this work, three criteria for selecting amino acid substitutions of the deamidation type in the *Bacillus gibsonii* alkaline protease (BgAP) are proposed and systematically studied in their influence on pH-dependent activity and thermal resistance. Out of 113 possible surface amino acids, 18 (11 Asn and 7 Gln) residues of BgAP were selected and evaluated based on three proposed criteria: (1) The Asn or Gln residues should not be conserved, (2) should be surface exposed, and (3) neighbored by glycine. "Deamidation" in five (N97, N253, Q37, Q200, and Q256) out of eight (N97, N154, N250, N253, Q37, Q107, Q200, and Q256) amino acids meeting all criteria resulted in increased proteolytic activity. In addition, pH activity profiles of the variants N253D and Q256E and the combined variant N253DQ256E were dramatically shifted towards higher activity at lower pH (range of 8.5–10). Variant N253DQ256E showed twice the specific activity of wild-type BgAP and its thermal resistance increased by 2.4 °C at pH 8.5. These property changes suggest that mimicking surface deamidation by substituting Gln by Glu and/or Asn by Asp might be a simple and fast protein reengineering approach for modulating enzyme properties such as activity, pH optimum, and thermal resistance.

Keywords Deamidation · Deamination · pH optimum · Protease · Surface charge engineering

Introduction

Subtilisins are important industrial enzymes as well as model enzymes for understanding structure function relationships (Bryan 2000; Maurer 2004). Extensive protein engineering approaches mainly accomplished by directed evolution, focused on altering the substrate specificity, general stability, and proteolytic activity are reported. Amino acid substitutions in over 50 % of the 275 amino acids of subtilisins are reported (Bryan 2000; Leisola and Turunen 2007). The focus of protease engineering has shifted in the last years towards engineering of proteases for novel and precise sequence specificity cleavage for the use in analytical, biotechnological, and therapeutic applications since a single protease molecule can inactivate, due to its catalytic turnover, numerous target proteins (Craik et al. 2011; Pogson et al. 2009). For example, subtilisin BPN' was through iterative modeling, mutagenesis, and kinetic analysis cycles, tailored to preferentially cleave phosphotyrosine peptides (2,500-fold enhanced relative to the wild type; Knight et al. 2007). Amino acid substitutions

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F. Jakob · R. Martinez · U. Schwaneberg (✉)
RWTH Aachen University, Lehrstuhl für Biotechnologie,
Worringerweg 1,
52074 Aachen, Germany
e-mail: u.schwaneberg@biotec.rwth-aachen.de

J. Mandawe · H. Hellmuth · P. Siegert · K.-H. Maurer
International Research Laundry & Home Care, Biotechnology,
Henkel AG & Co. KGaA,
40191 Düsseldorf, Germany

Present Address:
K.-H. Maurer
AB Enzymes GmbH,
Feldbergstr. 78,
64293 Darmstadt, Germany

responsible for the modified substrate specificity are mainly located in or around the specificity pockets and the active site (Di Cera 2008).

In contrast, the engineering of enzyme properties like activity, thermostability, and pH-dependent activity has been shown to be influenced by specific amino acid substitutions on the protein surface. Important factors are the hydrophobic/hydrophilic balance and the charge distribution on the surface. It was demonstrated that either the substitution of exposed hydrophobic amino acids against hydrophilic arginine or the addition of hydrophobic amino acids to the surface of a protein can result in increased protein stability (Sakoda and Imanaka 1992; Strub et al. 2004; Van den Burg et al. 1998).

Furthermore, the influence of charge distribution and charge–charge interactions on protein surfaces has been widely studied, including ionic strength-dependent activity, thermostability, and pH activity profile (De Kreij et al. 2002; Feller et al. 2010; Loladze et al. 1999; Russell and Fersht 1987; Sanchez-Ruiz and Makhatadze 2001). For subtilisins, the pH activity dependence on the protein surface charge was demonstrated by amino acid substitutions already in 1987 (Russell and Fersht 1987). Improvements in thermostability (up to 3–3.7-fold improved half-life times; Jaouadi et al. 2010) were achieved by single amino acid substitutions and by optimization of charge–charge interactions on the surface of serine proteases (Jaouadi et al. 2010; Loladze et al. 1999). Recently, Feller et al. studied the relationship between specific enzyme activity and protein charge of chymotrypsin-like serine protease from *Cellulomonas bogoriensis* in solution with different ionic strengths. Variants with increased positive charge showed a higher reaction rate at increased ionic strength, evidencing a clear influence of the protein charge on specific activity (Feller et al. 2010).

The amino acid residues asparagine (Asn) and glutamine (Gln) can be unstable under physiological conditions, undergoing a posttranslational nonenzymatic deamidation process, resulting in aspartic acid (Asp) and glutamic acid (Glu). As a result of this process, the number of amino acids with a negative charge in a protein increases. The deamidation process has been described extensively and probable mechanisms were proposed including the prediction of deamidation possibility in peptide sequences (Capasso et al. 1989; Catak et al. 2009; Bischoff and Schlüter 2012). Based on the deamidation process, a molecular clock hypothesis was proposed in which the nonenzymatic deamidation (half-life time of asparagine and glutamine residues under physiological conditions) serves as a regulator or "timer" for biological processes (Robinson and Robinson 2004).

First protein engineering studies by exchanging Asn to Asp at two positions in hen egg white lysozyme were performed by Kato et al. (1992). The exchange of Asn to Asp shifted the pH optimum from a neutral to a more acidic pH value (0.5 units shift). Thermostability of the deamidated variants was similar to the wild-type lysozyme and protease digestion studies suggested an increased accessibility and therefore a higher flexibility within the Asp-enriched lysozyme variants (Kato et al. 1992). This shows that changes in the protein charge profile resulting from amino acid substitutions of the deamidation type, especially in the surface, can lead to significant changes in the properties of the molecule, potentially leading to variants having more suitable thermal resistance, pH optimum, or specific activity, for specific applications.

In order to optimally select among all the potentially targetable Asn and Gln residues in *Bacillus gibsonii* (BgAP) protease (Siegert et al. 2009), we developed and validated a protein engineering strategy to determinate which substitutions would positively alter activity, thermal resistance, and pH optimum. After applying three criteria for selecting amino acid substitutions of the deamidation type (the Asn or Gln residues should not be conserved, they should be surface exposed and neighbored by Gly), the individual influences of 18 substitutions (11 Asn and 7 Gln) and combinations were studied and yielded first insights on how an increased negative surface charge shifts pH activity and increases thermal resistance as well as activity.

Material and methods

All chemicals were of analytical-reagent grade or higher quality and were purchased from Sigma-Aldrich (Taufkirchen, Germany) and AppliChem (Darmstadt, Germany). All enzymes were purchased from Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Frankfurt, Germany). Thermal cycler (Eppendorf Mastercyler proS, Hamburg, Germany) and thin-walled PCR tubes (Multi-ultra tubes, 0.2 mL, Carl Roth, Karlsruhe, Germany) were used in all PCRs. The PCR volume was always 25 μL. The amount of DNA in cloning experiments was quantified by using a NanoDrop photometer (Thermo Scientific NanoDrop 1000, Braunschweig, Germany).

B. gibsonii alkaline protease

The BgAP gene (GenBank entry GN111900.1) from *B. gibsonii* (DSM 14391) harboring the silent mutation t597a to delete an EcoRI restriction site, with the pre-pro-sequence and the *Bacillus* promoter was inserted into the pHY300PLK shuttle vector (Takara Bio Inc, Shiga, Japan). The generated construct was named pHYBgAP. BgAP gene is translated into a 270 amino acid mature protease with a size of approximately 27 kDa. As an alkaline protease, BgAP has an optimum pH of 11 (Siegert et al. 2009). The sequence numbering of the mature BgAP follows the

numbering of the *Bacillus lentus* alkaline protease (PDB: 1ST3; Goddette et al. 1992; see Fig. S1).

Sequence alignment

The sequence alignment of 15 BgAP-related proteases having an amino acid identity ranging from 79 to 31 % according to BLASTP (Altschul et al. 1997; PDB code: 1MPT [79 %], 1SVN [79 %], 1AH2 [78 %], 1C3L [56 %], 1SIB [55 %], 1SCJ [54 %], 1GNS [51 %], 1DBI [47 %], 1THM [45 %], 2XRM [41 %], 2Z2X [40 %], 2IXT [40 %], 2GKO [39 %], 2ID8 [37 %], 1WMD [31 %]) was performed with the multiple sequence alignment function of Clone Manager 9 Professional Edition (Sci-Ed software, Cary, USA). Residues were defined as not conserved with an identity of less than 60 % in the alignment.

Site-directed mutagenesis

Site-directed mutagenesis of BgAP was performed as described by Wang and Malcolm (1999) at 18 selected sites on BgAP in the pHYBgAP construct (see fig. S2). For the site-directed mutagenesis PCR (first stage, 98 °C for 30 s, 1 cycle; 98 °C, 10 s/55 °C, 30 s/72 °C, 2 min, 4 cycles; second stage, 98 °C for 30 s, 1 cycle; 98 °C, 10 s/55 °C, 30 s/72 °C, 3 min, 12 cycles; 72 °C for 3 min, 1 cycle), PhuS DNA Polymerase (2 U), 0.20 mM dNTP mix, and 0.2 μM of each primer together with template (20 ng; pHYBgAP) were used. Following the PCR, DpnI (20 U; New England Biolabs) was supplemented for template digestion and incubated overnight at 37 °C. The DpnI-digested PCR products were purified by using a NucleoSpin® Extract II Purification Kit (Macherey-Nagel, Düren, Germany) and transformed into *Escherichia coli* DH5α (Inoue et al. 1990). The *E. coli* DH5α mutants were cultivated (250 rpm, 37 °C, 16 h) and plasmids were isolated using a NucleoSpin Plasmid Kit (Macherey-Nagel) followed by transformation into protease-deficient *B. subtilis* DB104 strain (*nprR2 nprE18* and Δ*aprA3*) (Vojcic et al. 2012; Kawamura and Doi 1984) for extracellular BgAP expression.

Cell culture and expression

Expression of the generated protease variants in *B. subtilis* DB104 was performed in microtiter plates (Greiner, Frickenhausen, Germany). For expression buffered LB media (1 % (*w*/*v*) tryptone, 0.5 % (*w*/*v*) yeast extract and 1 % (*w*/*v*) sodium chloride, 17 mM potassium dihydrogen phosphate, and 72 mM dipotassium hydrogen phosphate) was used. The volume of 10 μL preculture (200 μL, 900 rpm, 37 °C, 18 h, and 70 % humidity) was used to inoculate the main culture (150 μL, 900 rpm, 37 °C, 24 h, and 70 % humidity). The protease fraction was separated from the cells by centrifugation (Eppendorf 5810R; 4 °C, 3,220×*g*, 20 min) and the obtained protease containing cell culture supernatant was used for analysis.

Skim milk detection system

A volume of 190 μL substrate solution (2 % skim milk, 100 mM Tris/HCl, pH8.6) was transferred in each well (flat bottom microtiter plates; Greiner, Frickenhausen, Germany) and 10 μL of cell culture supernatant was supplemented for quantifying proteolytic activity of secreted BgAP and BgAP variants. Protease activity was monitored by the decrease in absorbance with a microtiter plate reader (650 nm, 23 °C; TECAN Sunrise, Crailsheim, Germany). Decrease in absorbance at 650 nm (5 min incubation; 23 °C) was measured in absorbance units per min (AU/min).

Residual activity assay

Thermal inactivation of BgAP and BgAP variants in the cell culture supernatant was performed by incubating 25 μL of cell culture supernatant for 10 min at 60 °C (Eppendorf, Mastercyler proS). Residual proteolytic activity was determined by transferring 190 μL of substrate solution (2 % skim milk, 100 mM Tris/HCl, pH8.6, no additional $CaCl_2$ was supplemented) to 10 μL of the incubated protease containing supernatant. The residual activity was defined as the activity (AU/min) of the thermal incubated sample divided by the activity (AU/min) of sample without incubation in percent.

Native polyacrylamide gel electrophoresis

Native gels (GE Healthcare, PhastGel Gradient, 8–25 % acrylamide, 112 mM acetate, 112 mM Tris, pH6.4, Freiburg, Germany) and buffers (GE Healthcare, PhastGel Buffer Strips Native, 0.88 M L-alanine, 0.25 M Tris, pH8.8) were used for protein electrophoresis to analyze the charge differences of BgAP and its variants. The separation was accomplished in the PhastSystem (GE Healthcare, PhastSystem Automated Electrophoresis Development Unit) at 15 °C with reversed polarity of the electrodes. The protease bands were visualized using an activity overlay detection system with a gelatine photographic film (Paech et al. 1993).

Production and purification

BgAP and its variants were subcloned into a high-expression vector (provided by Henkel AG & Co. KGaA) and transformed into *B. subtilis* DB104. The expression of BgAP and its variants was accomplished in shaking flasks (500 mL) using MLBSP media (100 mL, 180 rpm, 37 °C, 48 h; Wilson et al. 1999). A clear supernatant was obtained after centrifugation (Eppendorf 5810R, 3,220×*g*, 40 min,

4 °C). The clear supernatant was dialyzed in HEPES buffer (9 L, pH7.8, 20 mM, 4 °C, 14 h) using a dialysis membrane with a 3.5-kDa cutoff (Spectrum Spectra/Por 3, Breda, Netherlands). The conductivity was, if required, adjusted to 1.2 mS/cm with demineralized water.

The dialyzed supernatant was loaded into an anion exchange chromatography column (GE Healthcare Q Sepharose Fast Flow; equilibrated with HEPES buffer (pH 7.8, 20 mM)) which was connected in series to a cation exchange column (GE Healthcare SP Sepharose Fast Flow; equilibrated with HEPES buffer (pH7.8, 20 mM)). The flow rate and pressure were monitored by an ÄKTA*explorer* (GE Healthcare). The anion exchange column acts as a "negative purification" and BgAP or its variants bound to the cation exchange column at pH7.8. For elution of BgAP, the anion exchange column was disconnected and a linear gradient of sodium chloride up to 1 M in HEPES buffer (pH7.8, 20 mM) was used to elute the absorbed proteins from the cation exchange column. The peak fractions were pooled together.

Protein concentration of purified BgAP variants was normalized by measuring the total protein concentration using the BCA protein assay kit (Thermo Scientific) and the Experion System (Experion Pro260 chip; Bio-Rad Laboratories Experion Automated Electrophoresis System, München, Germany).

pH activity profile of purified BgAP variants

Proteolytic activity on macromolecular substrate was assessed using azo dye-labeled collagen, as previously reported (Chavira et al. 1984). Azocoll (Merck/Calbiochem Azocoll MESH >100, Darmstadt, Germany) was used in final concentrations of 1 % (*w*/*v*) in 100 mM glycin and a pH range of 8.5–12 with adjustments made using 2 M NaOH and HCl. The proteolytic assay was performed in V-bottom microtiter plates (Corning, Wiesbaden, Germany) with protease concentrations of 40 nM. After reaction (Eppendorf Thermomixer comfort; 700 rpm, 25 min, 40 °C), the samples were incubated for 1 min on ice and centrifuged (Eppendorf 5810R; 3,220×*g*, 20 min, 4 °C). The absorbance of released azo dye in 100 μL supernatant was detected at 520 nm in a TECAN Sunrise using flat bottom microtiter plates (Greiner).

The complete digestion of 1 mg substrate resulted in an absorbance value at 520 nm of 1,506. One unit was defined as the enzyme activity producing an increase in absorbance of 1.0 min^{-1} at pH9.5 in 100 mM glycine/NaOH buffer. One unit corresponded to the digestion of 0.664 mg substrate.

Thermal resistance of purified variants

Thermal inactivation was monitored by incubating a volume of 25 μL BgAP and its variants (3.5 μg/mL; 20 min) at various temperatures (46–62 °C) in a thermal cycler (Eppendorf, Mastercyler proS) in glycin/NaOH buffer (100 mM, pH8.5 and pH10.5, no additional $CaCl_2$ was supplemented) and 250 mg/mL bovine serum albumin as an additive to prevent self-digestion. Residual activity was detected by using the skim milk detection system. The enzymes incubated on ice were considered to be the control and represent 100 % activity. The data were fitted to a sigmoidal curve in order to determine the temperature at which the initial activity value was decreased to 50 % (T_{50}) using GraphPad Prism (GraphPad software, San Diego, CA, USA).

Homology modeling and surface residue determination

The three-dimensional structure model for BgAP was generated using the homology model routine from Yet Another Scientific Artificial Reality Application (YASARA) (Krieger et al. 2002) based on the coordinates of a serine protease from *B. lentus* (PDB ID 1GCI; Kuhn et al. 1998). YASARA was used for visualization of the molecular structures. The surface residues of the generated BgAP model were determined using Discovery Studio 3.1 Visualizer (Accelrys, Inc., San Diego, USA) using the solvent accessibility dialog. A residue was defined as "exposed" or "buried" if the solvent accessibility was greater than 25 % or less than 10 % of the maximum solvent accessibility, respectively.

Results

The "Results" section is divided in to four parts: (a) Mutant generation and proteolytic activity of BgAP compared to BgAP variants with substitutions of the deamidation type (Asn → Asp, Gln → Glu), (b) analysis of the net charge in the generated variants, (c) pH-dependent activity profile of purified BgAP and variants (N253D, Q256E, and N253DQ256E), and (b) thermostability profiles of purified BgAP and variants (N253D, Q256E, and N253DQ256E).

Screening of proteolytic activity and thermal resistance

All generated variants were constructed as described under "Materials and methods." The targeted Asn and Gln residues were selected based on the following selection criteria: (1) surface exposed, (2) not conserved, and (3) neighbored by a glycine residue three amino acids up- or downstream. The third selection criterion is based on the posttranslational deamidation process which is described to be promoted when Asn or Gln is neighbored by glycine (Wright 1991; Kossiakoff 1988; Bischoff and Schlüter 2012). The third selection criterion reduces the number of Asn or Gln residues to sites with a high deamidation potential. Figure 1

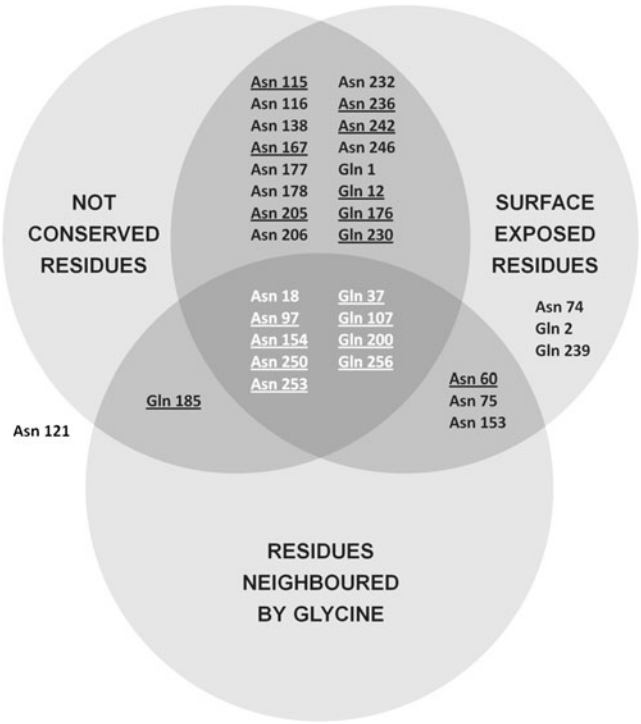

Fig. 1 Representation of all asparagine and glutamine residues present in BgAP assigned to the three defined selection criteria (not conserved residues, surface-exposed residues, and residues neighbored by glycine). The selected and by site-directed mutagenesis mutated positions are *underlined*

categorizes all Asn and Gln residues found in BgAP according to the three defined selection criteria. Nine positions (see Fig. 1) fulfill all three selection criteria and 16 sites fulfill the first two selection criteria. In order to access how important the third selection criterion is to identify high potential deamidation sites, we selected eight positions that fulfill all three criteria and eight positions that fulfill the selection criteria (1) and (2). The comparison of these two subsets of eight positions allows evaluating the importance of the third selection criterion. In addition, we selected one position (Asn 60) which fulfilled the selection criteria (1) and (3) and one position (Gln 185) which fulfilled the criteria (2) and (3). The underlined amino acid positions, a subset of in total 18 positions fulfilling at least two selection criteria, were subjected finally to site-directed mutagenesis and analyzed in microtiter plate format.

The variants N60D (surface exposed and neighbored by glycine) and Q185E (not conserved and neighbored by glycine) which fulfill only two selection criteria resulted in decreased proteolytic activity (N60D) or in an inactive variant (Q185E). For both variants, no residual activity was detected after thermal treatment (10 min, 60 °C; Table 1).

The selection criteria surface exposed and not conserved are fulfilled by 16 positions. A subset of eight positions was selected and substituted. Six variants (N205D, N236D, N242D, Q12E, Q176E, and Q230E) showed proteolytic activities in the range of wt-BgAP. Variant N115D was inactive and variant N167D showed a decrease in proteolytic activity of 23 % compared to wt-BgAP. In case of thermal resistance, two variants (N205D and Q12E) showed a decrease in residual activity of more than 16 %; however, the majority of the generated variants (N167D, N236D, N242D, and Q176E) were in the range of wt-BgAP (±10 %). An exception for this group is variant Q230E, which showed an increased thermal resistance of 22 % compared to wt-BgAP (Table 1).

Nine residues were identified which fulfill all three defined selection criteria (surface exposed, not conserved, and neighbored by glycine). Out of these nine targetable residues, eight variants were generated and tested. Variant Q107E showed 47 % decreased proteolytic activity compared to wt-BgAP and variants N154D and N250D were proteolytically as active as wt-BgAP. The remaining five variants (N97D, N253D, Q37E, Q200E, and Q256E) showed a more than 25 % increased proteolytic activity compared to wt-BgAP. The variants Q37E, N253D, and N97D showed with 36, 35, and 46 %, the strongest increase in proteolytic activity, whereas variants Q200E and Q256E showed a moderate increase in proteolytic activity (25 and 29 %). The aforementioned increase in activity was not observed in variants that did not fulfill all three defined selection criteria (Table 1). Regarding thermal resistance, the majority of the generated variants (N97D, N250D, Q37E, Q107E, and Q200E) had a comparable residual activity to wt-BgAP (±10 %), except variant N154D (12 % residual activity and less stable than wt-BgAP). Variants N253D and Q256E showed a moderately residual activity (84 and 71 %) and a significantly higher thermal resistance than the wt-BgAP (Table 1).

Variants N253D and Q256E were finally selected for detailed characterization due to their simultaneous increase in proteolytic activity and thermal resistance when compared to wt-BgAP. In addition, the double mutant N253DQ256E was generated to study possible cooperative or additive effects.

Net charge difference analysis of BgAP and its variants

The decreased positive net charge of N253D, Q256E, and N253DQ256E was confirmed by native polyacrylamide gel electrophoresis (Fig. 2) in which proteolytic activity was visualized using a gelatin film overlay (Paech et al. 1993).

Figure 2 shows distinct protein bands representing the different net charges of wt-BgAP, variants with Δ-1 (N253D or Q256E) and Δ-2 (N253DQ256E). The BgAP protease and variants are overall positively charged in the native gel at pH6.4 and the migration distance is decreased with reduced positive net charge.

Table 1 Proteolytic activities of BgAP and muteins before and after thermal treatment

	Relative proteolytic activity [% of wt-BgAP]	Residual proteolytic activity [% of initial activity]
WT	100±5 %	38±5 %
Surface exposed and neighbored by glycine		
N60D	32±14 %	Not active[a]
Not conserved and neighbored by glycine		
Q185E	Not active[a]	Not active[a]
Surface exposed and not conserved		
N115D	Not active[a]	Not active[a]
N167D	77±5 %	28±6 %
N205D	113±7 %	16±3 %
N236D	115±2 %	35±6 %
N242D	118±7 %	31±5 %
Q12E	114±8 %	13±1 %
Q176E	97±2 %	36±2 %
Q230E	107±8 %	50±5 %
Surface exposed, not conserved and neighbored by glycine		
N97D	146±3 %	44±2 %
N154D	107±4 %	12±5 %
N250D	113±8 %	45±6 %
N253D	135±4 %	84±4 %
Q37E	136±1 %	38±5 %
Q107E	53±8 %	32±5 %
Q200E	125±7 %	45±6 %
Q256E	129±4 %	71±3 %

Proteolytic activity was determined from the clear cell culture supernatant of *B. subtilis* DB104 cultures using the skim milk activity detection system (2 % (*w*/*v*) skim milk, 100 mM Tris/HCl pH8.6). The residual proteolytic activity (in percent) was defined as the activity (AU/min) of the thermal treated sample (10 min, 60 °C) divided by the activity (AU/min) of the untreated sample in percent. The variants are sorted according to the three selection criteria and measured in triplicates

[a]No activity detected under the defined conditions

pH-dependent activity profile of purified BgAP, N253D, Q256E, and N253DQ256E

Figure 3a shows the pH dependence of the proteolytic activity profiles of purified wt-BgAP, N253D, Q256E, and N253DQ256E which was determined using the azocoll proteolytic assay (pH range 8.5–12; 100 mM glycin/NaOH buffer).The azocoll assay is reliable over a broad pH range, whereas skim milk spontaneously hydrolyzes at high alkaline conditions. BgAP shows an activity profile with a maximum at pH11. The activity of BgAP at pH8.5 and pH12.0 was decreased to 50 and 83 %, respectively. Variants N253D and Q256E showed equal pH dependence, but differ from wt-BgAP since their pH activity optimum is shifted by 1 unit to pH10.0, without losing specific activity (Fig. 3a, b). The activity of N253D and Q256E is reduced to 71 and 72 % of the maximum at pH8.5 and pH12.0, respectively. The double mutant N253DQ256E shows also a shift on the pH activity optimum from pH11 to pH10, with comparable specific activity to wt-BgAP at pH11. The pH-dependent activity of N253DQ256E is reduced to 90 and 73 % at pH8.5 and pH 12.0, respectively (Fig. 3a, b). The pH activity profiles are in general shifted with reduced positive net charge to lower pH values leading to higher activities at reduced pH and reduced activity at alkaline pH.

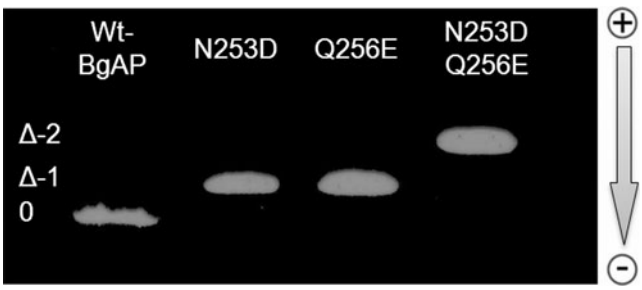

Fig. 2 Native polyacrylamide gel electrophoresis and gelatin film activity overlay of wt-BgAP and its variants (N253D, Q256E, and N253DQ256E) which differ in the positive net charge

Figure 3c shows that, normalized on the wt-BgAP activity, the residual pH activity dependence of the muteins N253D, Q256E, and N253DQ256E at a pH range from 8.5 to 12.0. Variants N253D and Q256E show a higher activity compared to wt-BgAP at pH values lower than 10. The wt-BgAP has at alkaline conditions pH >10, a superior proteolytic activity than N253D and Q256E.

The double mutant N253DQ256E shows at pH higher than 11 a nearly identical proteolytic activity compared to the single mutants. On the other hand, at lower pH values, the proteolytic activity exceeds significantly the values of the corresponding single mutants N253D and Q256E. At

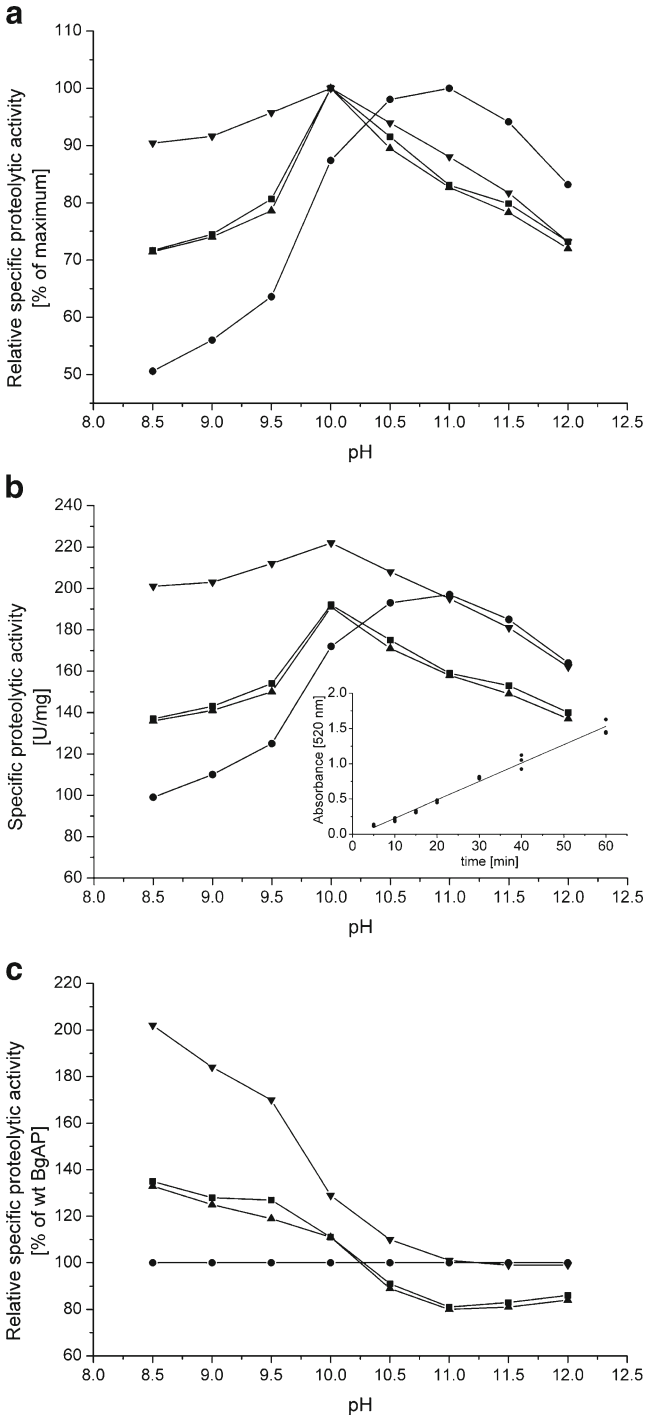

Fig. 3 **a** pH activity profile as percentage of maximum activity of each variant. **b** Specific proteolytic activity determined with the azocoll detection system (520 nm); inserted figure shows the linear detection range of the azocoll system. **c** Relative specific proteolytic activity as percentage of wt-BgAP activity at varied pH values. For activity measurements, 40 nM of wt-BgAP/muteins and 1 % (*w*/*v*) azocoll in 100 mM glycin/NaOH buffer (pH8.5 to 12) were employed. After 25 min incubation at 40 °C and 20 min centrifugation at 3,220×*g*, the absorbance of the supernatant was measured at 520 nm. Three measurements showed a standard deviation below 15 %; (*filled circle* wt-BgAP, *filled square* N253D, *filled triangle* Q256E, and *inverted filled triangle* N253DQ256E)

pH8.5, N253DQ256E shows a ~2.0-fold improvement in specific activity compared to wt-BgAP activity.

Thermostability profiles of purified BgAP, N253D, Q256E, and N253DQ256E

Figure 4a, b shows the residual activity of purified wt-BgAP, N253D, Q256E, and N253DQ256E after 20 min incubation at different temperatures and at two pH values (pH8.5 and 10.5). The generated variants showed a residual activity improvement of ~28 and ~37 % over wt-BgAP after being incubated at 53.2 and 55.4 °C (20 min; pH8.5). When incubated at pH10.5, the average residual activity of the BgAP muteins was improved by ~23 and 18 % compared to wt-BgAP (51.1 and 53.2 °C; 20 min). The T_{50} value is defined as the temperature at which the residual activity equals 50 % of the initial proteolytic activity after 20 min incubation at varied temperatures (gradient 46–62 °C) in glycin/NaOH buffer (100 mM, pH8.5 and 10.5).

The thermostability constant T_{50} at pH10.5 of wt-BgAP and the variants is 3–4 °C lower compared to pH8.5. At pH 8.5, the T_{50} value for N253D, Q256E, and N253DQ256E was found to be increased by 2.2, 2.2, and 2.4 °C, and in the case of pH10.5, the differences were determined to be 1.1, 1.5, and 1.3 °C, respectively (Table 2).

Discussion

Enzymatic and nonenzymatic deamidation has proven to have a significant influence on enzyme properties like activity, thermal resistance, and pH optimum (Capasso et al. 1989; Catak et al. 2009; Kato et al. 1987a; Kato et al. 1987b; Kato et al. 1992; Robinson and Robinson 2004). In the case of subtilisin proteases, there have been extensive reports on modifications in general stability and activity over the last decades by amino acid substitutions (Bryan 2000; Erwin et al. 1990; Li et al. 2012; Zhao and Arnold 1999). Out of the studied positions, three substitutions (Asn to Asp or Gln to Glu) at structurally similar positions have been previously reported to have an effect either in activity or stability on related subtilisins. In particular, the non-surface-located position N60 (N62 in subtilisin BPN') was reported to change substrate specificity towards cleavage of dibasic residues when exchanged by Asp (Ballinger et al. 1995), which can explain the reduced proteolytic activity observed in this study. Position Q200 (Q206 in subtilisin BPN') was reported to show an increased hydrolysis towards surface-bound substrates (1.4-fold increased) when Gln was exchanged by Glu (Brode et al. 1996). This result is similar to what was observed in this study (1.25-fold increased) even though different substrates were used. In the BgAP protease, residue N74 (N76 in subtilisin E) is located in the Ca

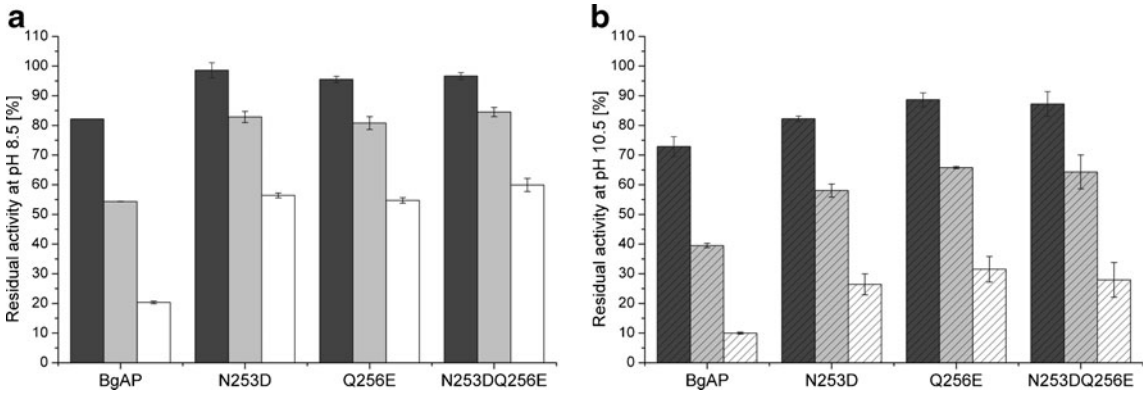

Fig. 4 Residual enzyme activity of wt-BgAP, N253D, Q256E, and N253DQ256E: (**a**) after being incubated at 51.1 °C (*black*), 53.2 °C (*gray*), and 55.4 °C (*white*) at pH8.5 for 20 min and (**b**) after being incubated at 49.2 °C (*black dashed*), 51.1 °C (*gray dashed*), and 53.2 °C (*white dashed*) at pH10.5 for 20 min. Heat incubation was performed with 4 μM of wt-BgAP and muteins (N253D, Q256E, and N253DQ256E) for 20 min in 100 mM glycin/NaOH buffer at pH8.5 and 10.5 and 250 mg/mL bovine serum albumin. Residual activity was measured with the skim milk detection system (2 % (*w*/*v*) skim milk, 100 mM Tris/HCl, pH8.6)

binding site, and it was shown that the substitution of Asn to Asp in this particular position has a strong effect on thermal resistance in subtilisin E by possibly enhancing calcium ion binding (Zhao and Arnold 1999). Since N74 is a conserved residue and not neighbored by a glycine, it was not targeted in this study (Fig. 1).

The three proposed selection criteria to target surface residues represent a straightforward and empirical sequence alignment-based strategy to identify Asn and Gln residues which can preferentially be targeted for deamidation by site-directed mutagenesis to generate variants with modified activity, thermal resistance, and/or shifted pH optimum. The first selection criterion of excluding Asn and Gln residues which are not exposed to the surface aims to avoid changing buried residues, which would have a higher impact in protein structure and probably decrease protein stability, as observed with variant Q185E which was inactive (Table 1). In a second step, non-conserved Asn and Gln residues are determined through sequence alignment with related subtilisin proteases to exclude structurally or catalytically important positions. In the third step, Asn and Gln residues are preferred which are neighbored by glycine, a deamidation-promoting amino acid (Bodanszky and Kwei 1978; Kossiakoff 1988; Teshima et al. 1991), within three amino acids up- or downstream on the sequence. The main aim of these criteria is to provide the subset of Asn and Gln that would yield a majority of active variants upon amino acid substitutions, from which the modified activity or stability properties can be identified. Thus, the targetable residues to introduce negative charges are dramatically reduced compared to the total amount of Asn and Gln residues in the protein. The protease BgAP consists of 270 amino acids, out of which 113 amino acids are surface exposed. The surface amino acids include 31 Asn and Gln residues, wherein 26 residues are not conserved. Only nine not conserved residues are neighbored by a glycine (three amino acids up- or downstream). Out of these nine variants, eight were generated and investigated. Five variants had an increased proteolytic activity at pH8.6. Two of the generated variants (N253D and Q256E) fulfilling all three selection criteria showed strong increased thermal resistance when compared to wt-BgAP (Table 1).

Out of the 16 Asn and Gln residues which lack the third criterion (neighbored by glycine), eight corresponding variants were generated, resulting in no variant with increased proteolytic activity and thermal resistance when compared to wt-BgAP. Only one variant (Q230E) had an increased residual

Table 2 The calculated T_{50} values of wt-BgAP, N253D, Q256E, and N253DQ256E are defined as the temperature at which 50 % of the initial activity is retained after 20 min incubation (100 mM glycin/NaOH, pH8.5 or pH 10.5, no additional $CaCl_2$ was supplemented)

	pH8.5		pH10.5	
Mutant	T_{50} [°C]	Increase [°C]	T_{50} [°C]	Increase [°C]
Wt-BgAP	53.4±0.3	–	50.5±0.1	–
N253D	55.6±0.3	2.2	51.6±0.2	1.1
Q256E	55.6±0.1	2.2	52.0±0.1	1.5
N253DQ256E	55.8±0.2	2.4	51.8±0.2	1.3

Average values of two independent measurements are shown and deviations are calculated from the fitted sigmoidal curves

activity compared to wt-BgAP. The two variants N60D (conserved) and Q185E (not surface exposed) showed decreased or no proteolytic activity when compared to wt-BgAP.

Due to the extent of improvements (Table 1), the positions N253D and Q256E and combination N253DQ256E were finally selected to detailed characterization in terms of: (a) activity increases (Fig. 3b, c), (b) influences on pH optima (Fig. 3a), and (c) thermal resistance improvements (Table 2 and Fig. 4). Substitutions N253D and Q256E are located on the same loop and N253DQ256E was generated to study possible cooperative or additive effects.

Variant N253DQ256E showed up to 2.4 °C increased T_{50} at pH8.5. No additive or cooperative effect could be observed when compared to the individual single mutants Q256E or N253D (Table 2 and Fig. 4). Wt-BgAP and the three deamidated variants showed at pH10.5 a smaller increase in thermal resistance compared to pH8.5, but still in a significant range. The determined residual activity is an indicator for thermal stability since the mature BgAP protease is not capable to refold correctly in absence of the propeptide (Siezen and Leunissen 1997).

The pH activity profiles were determined using an azo dye-labeled collagen as a substrate. Azo dye-labeled collagen is a complex protease substrate and close to application conditions in the detergent industry and therefore a more suitable choice than short chain peptides including chromogenic or flourogenic cleaving sites. The pH activity profiles for deamidated variants (N253D, Q256E, and N253DQ256E) were significantly shifted with a general trend towards higher proteolytic activity at lowered pH values resulting in a pH optimum shift from 11 to 10. The single variants (N253D and Q256E) show similar pH profile shifts. The combined variant N253DQ256E loses at pH8.5 only ~10 % of its activity, whereas as the single mutants have a ~30 % and the wt-BgAP has a 50 % reduced activity (Fig. 3). Figure 3c shows that the relative improvement in specific proteolytic activity for the double mutant exceeds the additive effects of the two single mutants. The latter result suggests that negative charges on surface could cause an increase in proteolytic activity through additive and/or cooperative effects.

De Kreij et al. studied the effects of modifying surface charges on the catalytic activity of a thermolysin-like protease (pH optimum ~6.8) by inserting or removing charges on the protein surface. Significant changes were observed in the activity of the thermolysin-like protease even when amino acid substitutions were as much as 25 Å apart from the active site (De Kreij et al. 2002). In our case, the finally studied amino acid substitutions (N253D, Q256E, and N253DQ256E) are located in a loop which is not close to the active site; however, activity as well as thermal resistance were increased. A similar observation has been reported for other proteases by Miyazaki et al., where the thermostability and low temperature activity of a psychrophilic subtilisin S41 were enhanced by directed evolution. The introduced mutations were, similar to our findings, far from the active site and located in the outer loops of the protease (Miyazaki et al. 2000). The substitutions generated in BgAP will likely cause electrostatic long-range interactions which are challenging to be addressed by computational modeling since structural changes that are related to increased protein stability are commonly described to cause reinforcement on the overall structural rigidity, which most likely goes along with a decrease in catalytic performance. The challenge for a molecular understanding lies in the build-up of a model in which the flexibility, as prerequisite for high activity, has to be maintained or improved around the active site, whereas global motions have to be reduced in regions at which unfolding occurs (Martinez et al. 2011).

In essence, we report a novel protein engineering approach, independent or additive to other methods, to quickly identify amino acid substitutions that could result in optimized protein properties like activity, thermal resistance, and pH optima through surface charge engineering and "simulated" deamidation by site-directed mutagenesis. Since the proposed criteria are in theory independent of the protein/enzyme class and taking in account the generality regarding the effect on surface changes on enzymes, it is likely that properties of enzymes from different classes can be modified using this approach.

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