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Reengineering of subtilisin Carlsberg for oxidative resistance

Abstract: Mild bleaching conditions by *in situ* production of hydrogen peroxide or peroxycarboxylic acid is attractive for pulp, textile, and cosmetics industries. The enzymatic generation of chemical oxidants is often limited by enzyme stability. The subtilisin Carlsberg variant T58A/L216W/*M221* is a promiscuous protease that was improved in producing peroxycarboxylic acids. In the current article, we identified two amino acid positions (Trp216 and Met221) that are important for the oxidative resistance of subtilisin Carlsberg T58A/L216W/*M221*. Site-saturation mutagenesis at positions Trp216 and Met221, which are located close to the active site, resulted in variants M4 (T58/W216M/*M221*) and M6 (T58A/W216L/M221C). Variants M4 (T58/W216M/*M221*) and M6 (T58A/W216L/M221C) have a 2.6-fold (M4) and 1.5-fold (M6) increased oxidative resistance and 1.4-fold increased k_{cat} values for peroxycarboxylic acid formation, compared with wild-type subtilisin Carlsberg.

Keywords: oxidation; peroxycarboxylic acid; promiscuity, protease; protein engineering.

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Introduction

Gentle bleaching under mild pH conditions can be achieved through *in situ* generation of low concentrations of peroxycarboxylic acids or hydrogen peroxide. Mild bleaching is especially attractive for cosmetics, therapeutics, washing, and disinfection applications (Rüsch gen. Klaas et al., 2002; Wieland et al., 2009). Peroxycarboxylic acid with a concentration around 1.5 mM is sufficient for bleaching in liquid detergent formulations. The common bleaching agent hydrogen peroxide is produced at high local concentrations by spontaneous decomposition of percarbonate and perborate combined with tetraacetylethylenediamine or nonanoyloxybenzenesulfonate (Wieland et al., 2009). Because of the hydrogen peroxide production conditions, it is not possible to maintain a low and constant level for mild bleaching. Peroxycarboxylic acids are more desirable in cleaning compositions because they are strong oxidizing agents with superior performance compared with hydrogen peroxide (Swern, 1949). Enzymatic *in situ* production of peroxycarboxylic acids by the hydrolysis of esters or amides in the presence of hydrogen peroxide is used in applications requiring mild bleaching conditions (Hofmann et al., 1992). Enzymatic generation of chemical oxidants is, in general, limited by the oxidative inactivation of the enzyme producer (Jori et al., 1968; Stauffer and Etson, 1969; Omenn et al., 1970; Kuroda et al., 1975; Simat and Steinhart, 1998). Among the 20 amino acids, Met, Cys, and Trp are most prone to oxidation (Dakin, 1906), especially if they are located close to the active site (Estell et al., 1985).

Systematic studies on the resistance of enzymes toward hydrogen peroxide were performed, for example, for chymotrypsin (Stauffer and Etson, 1969), lysozyme, ribonuclease A (Jori et al., 1968), and subtilisin from *Bacillus amyloliquefaciens* (Estell et al., 1985). Various mechanisms for understanding oxidative resistance have been proposed: (i) electronic changes such as sulfoxidation of Cys or Met close to or in the active sites and (ii) conformational changes in the protein structure. These conformational changes can be caused by the formation

of tyrosine dimers, reduction of side-chain hydrophobicity, introduction of more polar interactions, or changes in size and shape of amino acid residues (Kim and Stites, 2004). As a consequence, conformational changes can, for instance, reduce flexibility and, hence, enzyme activity. Alternatively, substrate access through the access channels can be blocked or the enzyme can denature after C-C bond breakage (Stauffer and Etson, 1969).

Subtilisin proteases are industrially important enzymes (e.g., laundry applications) and have become preferred targets in protein engineering (Maurer, 2004) and crystallography (Bryan, 2000). There are reports of a stabilized subtilisin from *B. amyloliquefaciens* in which its half-life was 24-fold increased ($t_{1/2}$ ~1 h compared with wild type $t_{1/2}$ ~2.5 min in 0.1 M H_2O_2) by substituting Met and Cys with Ala (Estell et al., 1985). Stauffer and Etson (1969) discovered that the oxidation of Met222 in subtilisin Carlsberg nearly inactivates the enzyme and that of the five Met, only Met222 was oxidized. Interestingly, the subtilisin-like protease KP-43 from *Bacillus* KSM exhibits an increased tolerance toward hydrogen peroxide (Nonaka et al., 2004) despite having two Met residues located near the active site.

The catalytic promiscuity of the proteases generating the peroxycarboxylic acids is an attractive side activity of subtilisin Carlsberg. Recently, a subtilisin Carlsberg variant T58A/L216W/*M221* with increased peroxycarboxylic acid production and decreased proteolytic activity was reported along with a molecular dynamics study on the second tetrahedral intermediate (Wieland et al., 2009; Lee et al., 2010).

In this study, we identified Trp216 and Met221 as the important sites for the oxidative resistance of subtilisin Carlsberg. Residues Trp216 and Met221 are located close to the active site, and site-saturation mutagenesis of both positions with subsequent recombination of beneficial substitutions resulted in subtilisin Carlsberg variants with increased peroxycarboxylic acids production and oxidative resistance.

Results

Optimization of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*NA-based screening system

The proteolytic activity of neutral and alkaline proteases can be determined and quantified using an artificial substrate, succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*NA (suc-AAPF-*p*NA) (DelMar et al., 1979). The T58A/L216W/*M221* variant was used as the parent due to increased peroxycarboxylic acid production and to optimize the screening conditions [proteolytic activity of the variants with and without incubation in peroxyacetic acid (PAA)]. The residual activity of the T58A/L216W/*M221* variant was 23% after 20 min of incubation in the presence of PAA (1.7 mM) (Figure S1). A concentration of 1.7 mM PAA was finally used in all screening experiments with a 20-min incubation time at room temperature.

Diversity generation

The structural analysis of subtilisin Carlsberg (Protein Data Bank entry 1YU6) was performed to select amino acid residues that are susceptible to oxidation. The visual inspection of the whole protein suggested that positions Trp216 and Met221, which are located near the active site are prone to oxidation (the closest distance between heavy atoms are 6.0 and 3.5 Å, respectively; Figure S2). The site-saturation mutagenesis libraries at positions Trp216 and Met221 were generated, and screening yielded four variants with increased oxidative resistance M1 (T58A/L216W/M221S),

Figure 1 Genealogic tree of subtilisin Carlsberg variants.
The variant T58A/L216W/*M221* was patented (Wieland et al., 2009) and provided by Henkel AG & Co KGaA (Düsseldorf, Germany). T58A/L216W/*M221* was used as the starting variant for the current site-saturation (SSM) and site-directed (SDM) studies. Amino acid positions Trp216 and Met221 were identified as the key positions for oxidative resistance of subtilisin Carlsberg.

M2 (T58A/L216W/M221C), M3 (T58A/W216L/*M221*), and M4 (T58A/W216M/*M221*). The beneficial substitutions were subsequently combined in variants M5 (T58A/W216L/M221S), M6 (T58A/W216L/M221C**)**, and M7 (T58A/W216M/M221C) (Figure 1) by site-directed mutagenesis to explore cooperative effects.

Table 1 shows the resistance of all generated variants (T58A/L216W/*M221* to M7) in the supernatants of *Bacillus subtilis* DB104 in the presence of PAA (1.7 mM).

The variants generated by combining the beneficial mutations (M5–M7) have a comparable oxidative resistance to variants M1–M4 having a single substitution. Results on the proteolytic activity indicate a cooperative effect in contrast to the oxidative resistance values (Table 1). For instance, the combination of W216M/M221C in M7 significantly increases the proteolytic activity (120% in M2 compared with 280% in M7). Slight increases in proteolytic activity can also be observed for M5 (40% in M1 compared with 60% in M5) and for M6 (120% in M2 compared with 140% in M6).

Oxidative resistance: inactivation by PAA (PS_{50} value)

The concentration of PAA at which the proteolytic activity of subtilisin Carlsberg and variants is decreased to 50% (20-min incubation) was defined as PS_{50}. The range of PAA concentration varied from 0 to 4 mM for all 10 purified subtilisins. Figure 2 shows the residual activities at 0, 1, 2, and 4 mM PAA of the wild type, parent (T58A/L216W/*M221*), and variants M1, M4, M5, and M6. Variants M2, M3, and M7 showed a low resistance to PAA at concentrations higher than 1 mM. The values of the residual activities for subtilisin Carlsberg, parent, and variants M1, M4, M5, and M6 were used to calculate the PS_{50} value as described in the materials and methods section. PS_{50} values from the exponential single curve fitting (see Figure S3 in the supplementary material) are presented in Table 2.

Variants M4 (T58A/W216M/*M221*) and M5 (T58A/W216L/M221S) have an increased PS_{50} for 5.1- and 3.5-fold compared with the parent (T58A/L216W/*M221*) followed by increase of 3.2- and 3.0-fold for variants M1 (T58A/L216W/M221S) and M6 (T58A/W216L/M221C), respectively.

Variant	Residual activity (%)[a]	Proteolytic activity (%)
Parent (T58A/L216W/*M221*)	23	100
M1 (T58A/L216W/M221S)	80	40
M2 (T58A/L216W/M221C)	75	120
M3 (T58A/W216L[b]/*M221*)	80	110
M4 (T58A/W216M/*M221*)	75	130
M5 (T58A/W216L[b]/M221S)	59	60
M6 (T58A/W216L[b]/M221C)	70	140
M7 (T58A/W216M/M221C)	80	280

Table 1 Proteolytic and residual activity of variants generated using site-saturation and site-directed mutagenesis.
Activities are measured in cell-free supernatants (nonpurified) using suc-AAPF-*p*NA-based assay. Variants having the wild-type residue at position 221 are in italic.
[a]The residual activity was determined as a ratio of protease activity after incubation with PAA divided by protease activity in the absence of PAA. The residual activity was used as a selection criterion for oxidative resistance.
[b]Wild-type subtilisin Carlsberg has a Leu residue at position 216.

Kinetic characterization of subtilisin Carlsberg variants

Proteolytic activity determination using suc-AAPF-*p*NA as a substrate

The kinetic parameters for the proteolytic activity of the selected and purified variants with increased PS_{50} value were determined by using the suc-AAPF-*p*NA assay (DelMar et al., 1979). Table 3 summarizes the kinetic parameters of the variants. Variant M6 showed a k_{cat} value comparable to subtilisin Carlsberg, whereas the k_{cat} values were reduced (5.7–2.2 times) in variants M1, M4, and M5 and the parent (T58A/L216W/*M221*).

Perhydrolytic activity determination using methylbutyrate as a substrate

The kinetic parameters for the perhydrolytic activity using methylbutyrate as a substrate were determined using the APPC detection system (100 mM H_2O_2; Despotovic et al., 2012) for subtilisin Carlsberg, parent, and variants (M1, M4, M5, and M6). Variants M1, M4, M5, and M6 were selected for characterization because of their increased PS_{50} values (Table 3).

The parent T58A/L216W/*M221* showed the highest perhydrolytic activity, which is two times higher when compared with wild-type subtilisin Carlsberg (*L216*/*M221*) and 1.4 times higher when compared with M4 (T58A/W216M/*M221*) and M6 (T58A/W216L/M221C). However, the oxidative resistance of the parent is lower

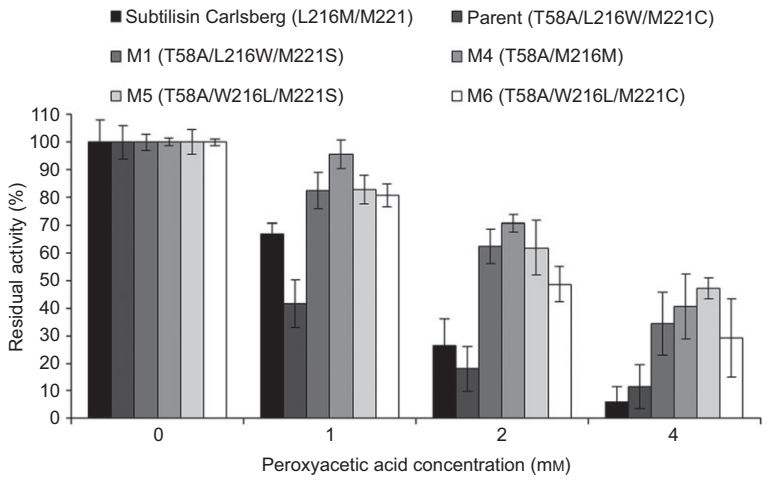

Figure 2 Residual activity of subtilisin Carlsberg (black), parent (darker gray), and variants M1 (dark gray), M4 (medium gray), M5 (light gray), and M6 (white) at various concentrations of PAA (0–4 mM) in sodium phosphate buffer (100 mM, pH 7.5, 20-min incubation time). The reported values are based on triplicate measurements.

than for variants M4 (5.1-fold) and M6 (3.0-fold). In essence, variant M4 (T58A/W216M/*M221*) is 'the best' compromise in terms of production of peroxycarboxylic acids and oxidative resistance.

Variants	PS_{50} (mM)
Subtilisin Carlsberg (*L216/M221*)	1.2
Parent (T58A/L216W/*M221*)	0.6
M1 (T58A/L216W/M221S)	1.9
M4 (T58A/W216M/*M221*)	3.1
M5 (T58A/W216L[a]/M221S)	2.1
M6 (T58A/W216L[a]/M221C)	1.8

Table 2 Calculated PS_{50} values for subtilisin Carlsberg, parent (T58A/L216W/*M221*), and variants M1, M4, M5, and M6 using single exponential fitting (see Figure S3).
Amino acids at positions 216 and 221 with wild-type residue are italicized.
[a]Wild-type subtilisin Carlsberg has a Leu residue at position 216.

Computational study of subtilisin variants

The stabilization of the H-bond network in the second tetrahedral intermediate is crucial for the perhydrolytic reaction of subtilisin protease (Lee et al., 2010). Therefore, we generated several minimal models by simulated annealing of the subtilisin variants according to the procedure given in the materials and methods section. Three different conformations resulting in three possible hydrogen bond networks were identified by measuring H-bond distances in the second tetrahedral intermediate state of the perhydrolytic reaction. Table 4 summarizes the results on hydrogen bond distances and stabilization energies of the subtilisin Carlsberg wild type (*L216/M221*), parent (T58A/L216W/*M221*), and variants M4 (T58A/W216M/*M2211*) and M6 (T58A/W216L/M221C). In the parent (T58A/L216/*M221*) and variant M4 (T58A/W216M/*M221*), the favored conformer is His63Nε-H-OGSer220 (promotes

Variant	Suc-AAPF-*p*NA		Methylbutyrate	
	K_M (mM)	k_{cat} (min^{-1})	K_M (mM)	k_{cat} (min^{-1})
Subtilisin Carlsberg (*L216/M221*)	0.59±0.06	10 353±342	117±26	124±13
Parent (T58A/L216W/*M221*)	0.15±0.02	1983±43	122±36	258±38
M1 (T58A/L216W/M221S)	0.60±0.08	1809±70	153±57	37±7
M4 (T58A/W216M/*M221*)	0.43±0.07	4374±79	139±42	179±28
M5 (T58A/*W216L*[a]/M221S)	1.26±0.09	4646±149	164±60	49±9
M6 (T58A/*W216L*[a]/M221C)	0.98±0.15	11 551±656	160±56	175±33

Table 3 Kinetic parameters of subtilisin Carlsberg, parent, and variants (M1, M4, M5, and M6) for proteolytic activity determined with the suc-AAPF-*p*NA detection system and perhydrolytic activity determined with methylbutyrate.
The reported values are the average values of triplicate measurements. Amino acids at positions 216 and 221 with wild-type residue are italicized.
[a]Wild-type subtilisin Carlsberg has a Leu residue at position 216.

Variants	Nε-H-OG (Å)	Nε-H-O2 (Å)	ΔE[a] (kJ/mol)
Subtilisin Carlsberg (*L216M221*)	2.6	1.9	7.8
Parent (T8A/L216W/*M221*)	2.0	2.5	-3.0
M4 (T58A/W216M/*M221*)	2.1	2.5	-12
M6 (T58A/W216L[b]/M221C)	1.9	2.7	5.5

Table 4 Hydrogen bond distances and stabilization energies for subtilisin Carlsberg, parent (T58A/L216W/*M221*), M4 (T58A/W216M/*M221*), and M6 (T58A/W216L/M221C).
Amino acids at positions 216 and 221 with wild-type residue are italicized.
[a]$\Delta E = E_{(N\varepsilon\text{-H-OG})} - E_{(N\varepsilon\text{-H-O2})}$.
[b]Wild-type subtilisin Carlsberg has a Leu residue at position 216.

perhydrolytic reaction); in subtilisin Carlsberg and variant M6 (T58A/W216L/M221C), the favored conformation is His63Nε-H-O2 ('backward' reaction; release of hydrogen peroxide and carboxylic acid).

Discussion

Subtilisin Carlsberg is the first example of a promiscuous protease that can catalyze perhydrolysis besides its natural proteolytic activity. The variant subtilisin Carlsberg T58A/L216W/*M221* was patented by Henkel AG & Co. KGaA (Wieland et al., 2009) due to increased peroxycarboxylic acid production (Table 3).

Three amino acids that govern peroxycarboxylic acid formation and oxidative resistance were found at position 216 (L, W, M) and position 221 (S, C, M) (Table S1). The ratios of $PS50_{variants}$ to $PS50_{subtilisin\ Carlsberg}$ (PS_{50}v/PS_{50}sC) were used in this work as benchmark to compare the oxidative resistance of the subtilisin Carlsberg variants against wild-type.

The beneficial substitutions leading to increased resistance against PAA in variant M5 (T58A/W216L/M221S) comprise an exchange from Trp and Met to Leu and Ser, which are less prone to oxidation. The substitution of Met221 in the parent (T58A/L216W/*M221*) with Ser in variant M1 (T58A/L216W/M221S) shows an expected increase in oxidative resistance and proves that Met221 is a key residue for oxidative resistance in subtilisin Carlsberg.

M6 (T58A/W216L/M221C) differs from the subtilisin Carlsberg wild type (*T58/L216/M221*) with the substitution M221C, which likely contributes to M6's increased oxidative resistance (1.5-fold). Similarly, for subtilisin from *B. amyloliquefacines*, a slower oxidation rate was reported due to an exchange of Met222 to Cys (Estell et al., 1985).

Solvent accessibility of Cys and Met is a parameter for their oxidative sensitivity. In *Staphylococcal* nuclease, two buried and two solvent-exposed Cys were introduced to study the destabilization effect on a nuclease structure caused by hydrogen peroxide. It has been shown that the solvent-exposed Cys has a higher sensitivity to oxidation compared with the buried one (~2.0-fold increased oxidation rate) (Kim and Stites, 2004). The solvent accessibility of the Cys221 side chain in variant M6 (T58A/W216L/M221C) was calculated to be 6.6%. Cys221 should therefore be highly protected and slowly oxidized.

Surprisingly, variant M4 (T58A/W216M/*M221*), despite having two Met residues susceptible to oxidation (Met216 and Met221), shows the highest PS_{50} value. The oxidative resistance of variant M4 (T58A/W216M/*M221*) compared with subtilisin Carlsberg wild type (PS_{50}v/PS_{50}sC ratio) is increased by 2.6-fold. Stadtman et al. (2003) proposed that a combination of two Met residues near the active site can cause an increase in hydrogen peroxide resistance if one methionine acts as a suicide antioxidant, which prevents the oxidation of the catalytically important Met residue. The more solvent-exposed methionine will be preferentially oxidized to methionine sulfoxide, which further reduces the solvent accessibility of the catalytically important methionine and reduces its oxidation rate. The analysis of the generated three-dimensional model of variant M4 (T58A/W216M/*M221*) indicated that the catalytically important residue Met221 is buried (side-chain solvent accessibility, 0.39%), whereas residue Met216 is highly solvent exposed (side-chain solvent accessibility 44.85%; Figure 3).

KP-43, a subtilisin-like alkaline protease from *Bacillus* KSM (Nonaka et al., 2004), shows a naturally high tolerance toward hydrogen peroxide (up to 0.88 M). A structural comparison of KP-43 (Nonaka et al., 2004) and the subtilisin Carlsberg variant M4 (T58A/W216M/*M221*) revealed that KP-43 has two methionines, Met251 and Met256, at the structurally identical positions of M4 (Met216 and Met221). Figure 4 shows in a structural overlay the orientation of both Met residues in the X-ray structure of KP-43 and in the homology model of variant M4 after energy minimization (three annealing cycles from 298 to 5 K; YASARA 11.9.8, Force field AMBER03; Krieger et al., 2002). The structural comparison of KP-43 with M4 in Figure 4 presents a further indication that a 'two methionine pair' around the catalytic Ser220 represents a general principle to improve oxidative resistance in alkaline proteases.

Substitution of amino acid residues located near the active site affected the rate of peroxycarboxylic acid production. The parent variant T58A/L216W/*M221* remained the variant with the highest production of

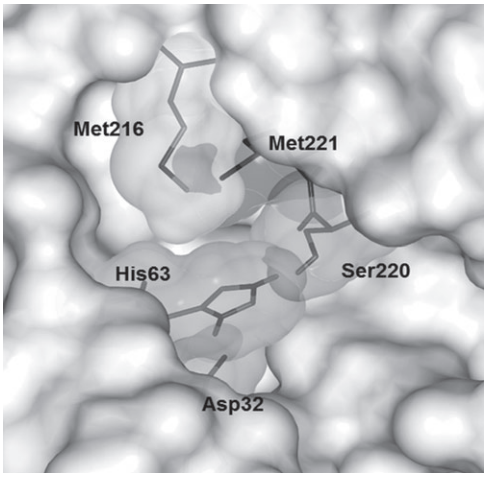

Figure 3 Structural models of the subtilisin Carlsberg variant M4 (T58A/W216M/*M221*) and the catalytic site (Asp32, His63, and Ser220).
Positions Met216 and Met221 are located close to the catalytic center. The surface representation of Met216 (light gray) and Met221 (dark gray) shows the difference in solvent-exposed surfaces.

peroxycarboxylic acids. Molecular simulation studies on this variant revealed a stabilization effect of Trp216 on the second tetrahedral intermediate by hydrogen bonding between Nε-H in Trp216 and the hydroxyl oxygen of the second tetrahedral intermediate state (Lee et al., 2010). Variants M1 (T58A/L216W/M221S) and M5 (T58A/W216L/M221S) showed a decreased production of peroxycarboxylic acids. The kinetic data for peroxycarboxylic acid production showed that position Met221, located next to the catalytic residue Ser220, is crucial for perhydrolytic and proteolytic activates. Substitution from hydrophobic Met to hydrophilic Ser in variants M1 (T58A/L216W/M221S) and M5 (T58A/W216L/M221S) reduces the rates of perhydrolytic (M1, 3.3-fold; M5, 2.5-fold) and proteolytic activities (M1, 5.7-fold; M5, 2.2-fold) when compared with the subtilisin Carlsberg wild type.

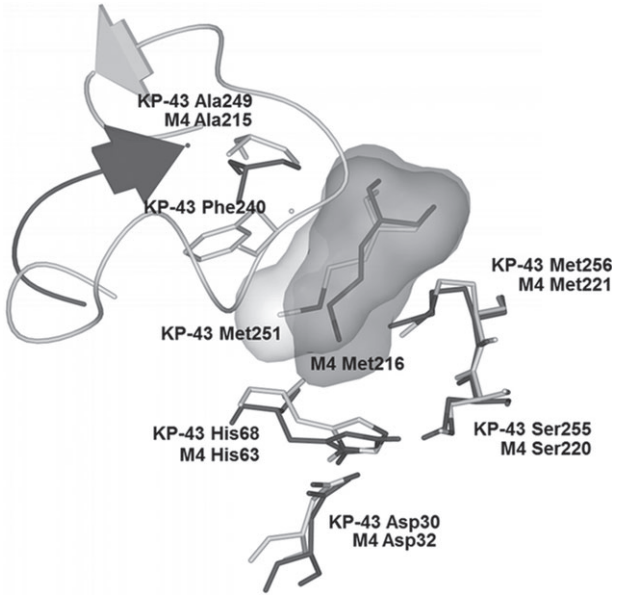

Figure 4 Structural overlay of the stick models of KP-43 subtilisin-like protease and the subtilisin Carlsberg variant M4 (T58A/W216M/*M221*).
The subtilisin Carlsberg variant M4 (T58A/W216M/*M221*) is shown in dark gray. The KP-43 subtilisin-like protease with PDB accession code 1WMD is shown in light gray. Structural alignment revealed a 32.6% sequence identity between both proteases.

Trp216 was reported to be a key residue for peroxycarboxylic acid formation (Lee et al., 2010) and M1 harbors L216W/M221S, whereas M5 harbors W216L/M221S. Comparison of k_{cat} values for perhydrolysis of M1 (37 min^{-1}) and M5 (49 min^{-1}) indicate only a low peroxycarboxylic acid production rate despite the presence of a Trp216 in M1. High peroxycarboxylic acid production rates are only reported when Trp216 is accompanied by M221 (parent). Therefore, one could conclude that Trp216 can only stabilize the second tetrahedral intermediate in the presence of Met221.

The parent (T58A/L216W/*M221*) has a 2-fold increase and variants M4 (T58A/W216M/*M221*) and M6 (T58A/W216L/M221C) have a 1.4-fold increase in peroxycarboxylic acid production when compared with the subtilisin Carlsberg wild type. Based on a previous study (Lee et al., 2010), the hydrogen bond networks were investigated in the second tetrahedral intermediate in subtilisin Carlsberg, parent (T58A/L216W/*M221*), and variants M4 (T58A/W216M/*M221*) and M6 (T58A/W216L/M221C) (see computational study in the materials and methods section and Tables S2–S5). In addition to hydrogen bond formation, the preferred conformational states of the local environment in the second tetrahedral intermediate were investigated by calculating the stabilization energies of residues Asp32, His63, Asp154, Leu/Trp216, Ser220, and Met221 (Table 4). The differences in the stabilization energy of the variants in the two conformational states are given in Table 4. The hydrogen bond length (Nε-H-*OG*; Table 4) is an indicator of the strength of the hydrogen bond network in a second tetrahedral intermediate conformation that will lead to peroxycarboxylic acid formation (Figure 5). The hydrogen bond length (Nε-H-*O2*; Table 4) is an indicator of the strength of the hydrogen bond network in a conformation that will lead to an unwanted hydrogen peroxide and carboxylic acid formation (Figure 5). The shortened Nε-H-OG bond lengths from subtilisin Carlsberg to the parent and variants M4 and M6 (Table 4) indicate a promotion of the peroxycarboxylic

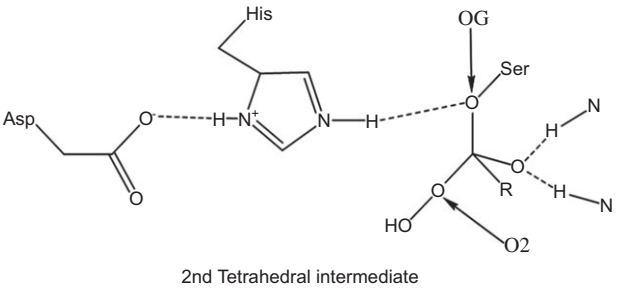

Figure 5 Schematic representation of the catalytic triad and the second tetrahedral intermediate of the perhydrolytic reaction. The formation of hydrogen bond between His Nε-H and the oxygen of Ser (OG atom) leads to the release of the peroxycarboxylic acid. In the case of bond formation between His Nε-H and the oxygen from the peroxy group (O_2), an unwanted hydrolysis to hydrogen peroxide and carboxylic acid is promoted.

formation, which is supported by elongated bond lengths (Nε-H-O2) of the unwanted hydrolysis reaction. Stabilization energy values support the bond length calculations for the wild-type subtilisin Carlsberg (*L216/M221*), parent (T58A/L216W/*M221*), and M4 (T58A/W216M/M221), whereas in M6 (T58A/W216L/M221C), the energy stabilization values favor the conformer, which leads to an undesired hydrolysis reaction. The latter discrepancy between our model and the observed perhydrolysis rates might be attributed to larger conformational rearrangements in the environment of the catalytic residues Ser221 and His63. The conformational changes are most likely occurring due to the smaller sizes of Cys and Met, which is not reflected in our model using a simulated annealing procedure starting from the subtilisin Carlsberg wild-type geometry.

In summary, we proved that subtilisin proteases can be simultaneously tailored for increased perhydrolytic activity and increased oxidative resistance in the presence of peroxycarboxylic acids. The latter can be achieved if amino acids that are prone to oxidation and close to the active site are substituted with less sensitive ones. An in-depth analysis leads to the hypothesis that a couple of methionine residues surrounding the catalytic Ser220 residue might represent a general principle to improve oxidative resistance in subtilisin-like proteases despite their oxidative stability. One methionine will thereby sacrifice itself by acting as a 'suicide' antioxidant reducing the solvent accessibility of the catalytically important methionine. In terms of performance, variant M4 (T58A/W216M/*M221*) seems to be the 'best' compromise between peroxycarboxylic acid production and oxidative resistance.

Materials and methods

All chemicals were of analytical reagent grade or higher quality and purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) or AppliChem (Darmstadt, Germany). The PAA solution was purchased from Sigma-Aldrich (32% PAA). All enzymes were purchased from New England Biolabs GmbH (Frankfurt, Germany) and Fermentas GmbH (St. Leon-Rot, Germany).

The thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) and thin-wall PCR tubes (Multi-ultra tubes; 0.2 ml; Carl Roth GmbH, Karlsruhe, Germany) were used in all PCRs. The PCR volume was always 50 µl. The amount of DNA in cloning experiments was quantified using a NanoDrop photometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA). An Infinite M1000 (Tecan Group AG, Zürich, Switzerland) plate reader was used for the detection of fluorescence, and a Sunrise microtiter plate reader (Tecan Group AG) was used for absorbance measurements.

Subtilisin Carlsberg (*L216/M221*) and variant T58A/L216W/*M221* were kindly provided by Henkel AG & Co. KGaA (Düsseldorf, Germany).

Cloning of subtilisin Carlsberg and variant T58A/L216W into the shuttle vector pHY300PLK

Subtilisin Carlsberg (*L216/M221*) and its variant T58A/L216W/*M221* (along with its pre-pro-sequence and promoter) were cloned into the pHY300PLK shuttle vector (Takara Bio Inc., Shiga, Japan) as previously described (Despotovic et al., 2012).

Site-saturation and site-directed mutagenesis at positions Trp216 and Met221

Site-saturation mutagenesis of the subtilisin Carlsberg variant T58A/L216W/*M221* at positions Trp216 and Met221 were performed according to a standard site-saturation mutagenesis protocol (Wang and Malcolm, 1999). The list of primers, PCR mixture, and two-step PCR program are described in the supplementary material (Tables S6–S10).

Cultivation and expression in 96-well microtiter plates

Cultivation and expression in 96-well plates (flat-bottomed polystyrene plates; Greiner Bio-One GmbH, Frickenhausen, Germany) were performed as previously described (Despotovic et al., 2012).

Screening procedure

An spectrophotometric suc-AAPF-*p*NA assay was used to determine the activity of the protease before and after incubation with PAA (32 wt% in diluted acetic acid; Sigma-Aldrich, Taufkirchen,

Germany). The proteolytic activity was determined by the quantification of the release of free *p*-nitroaniline (*p*NA) at 410 nm (DelMar et al., 1979). The concentration of the peptide substrate was determined using an extinction coefficient of ε_{410}=8800/M×cm (DelMar et al., 1979). The supernatant of *B. subtilis* DB104 cells was incubated in the PAA solution (final concentration, 1.7 mM; 20 min). The release of yellow *p*NA was initiated by the addition of an 'incubated' supernatant to the suc-AAPF-*p*NA solution (0.22 mM) in sodium phosphate buffer (100 mM, pH 7.5; final volume 100 µl) and monitored as increase of absorbance per minute at 410 nm (5-min reaction time; 30°C). The proteolytic activity of the 'treated' supernatant was subsequently determined and compared with the activity of the 'untreated' supernatant, and the residual activity was calculated. Residual activity (%) was defined as the activity of the 'treated' sample in absorbance unit (AU) per minute divided by the activity of the 'untreated' sample, multiplied by 100, and this was taken as the indicator of oxidative resistance of protease variants. The standard deviation of proteolytic activity for 96 clones was calculated from the change of absorbance intensity per minute (ΔAU/min).

Expression and purification of subtilisin Carlsberg and variants

The expression of subtilisin Carlsberg and its variant in shaking flask, purification by anion, and subsequent cation exchange chromatography were performed as previously described (Despotovic et al., 2012).

Determination of kinetic parameters for suc-AAPF-*p*NA as an artificial proteolytic substrate

After the purification, the kinetic characterization of subtilisin Carlsberg and variants (M1, M2, M3, M4, M5, M6, and M7) was performed using the synthetic tetrapeptide suc-AAPF-*p*NA (0.05–3 mM) in sodium phosphate buffer (100 mM, pH 7.5). The proteolytic reaction was initiated by the addition of purified protease or variant (0.3, 0.5, and 1.4 nM) to the suc-AAPF-*p*NA substrate solution (0.05–3 mM) and the increase of absorbance was recorded (410 nm; for 5 min). Released *p*NA concentration was calculated using ε_{410}=8800/M×cm as recommended by the manufacturer (Sigma-Aldrich). The initial velocity data were fitted using GraphPad Prism software (GraphPad, San Diego, CA, USA; hyperbolic fitting). The k_{cat} value was calculated from the ratio of V_{max} and protease concentration.

Oxidative resistance: inactivation by PAA (PS_{50} value)

PAA inactivation was monitored by incubating the enzyme (0.5 nM) in different concentrations of PAA (0–20 mM) in sodium phosphate buffer (100 mM, pH 7.5). Residual activity was measured by adding an 'incubated' enzyme solution into the suc-AAPF-*p*NA (2 mM) solution; the increase in absorbance was recorded (410 nm, for 2.5 min). Residual activity was determined for subtilisin Carlsberg and variants as a ratio of activity after the incubation with PAA divided by the activity in the absence of PAA and was taken as an indicator of oxidative resistance. PS_{50} values were calculated using GraphPad software, with single exponential fitting.

Determination of kinetic parameters for peroxybutyric acid production by methylbutyrate conversion as recently reported (Despotovic et al., 2012)

The k_{cat} and K_M values were determined from initial velocity data measured as a function of methylbutyrate concentration at 30°C in 96-well microtiter plates (black, flat-bottomed polystyrene plates; Greiner Bio-One GmbH). After 10 min of preincubation at 30°C, the perhydrolytic reaction was initiated by the addition of a purified protease or a protease variant (0.93 nM) to the substrate solution containing methylbutyrate (10–200 mM), hydrogen peroxide (100 mM), sodium bromide (100 mM), and 7-(4′-aminophenoxy)-3-carboxycoumarin (APCC) (0.5 mM) in sodium phosphate buffer (100 mM; pH, 7.5; final volume, 100 µl). Fluorescence was measured at 360 nm (ex.) and 465 nm (em.) using an Infinite M1000 (gain factor, 100; 30°C; Tecan Group AG). The initial velocity data were fitted using GraphPad Prism software (GraphPad software; hyperbolic fitting). k_{cat} values were calculated from the ratio of V_{max} and protease concentration.

Modeling studies

Analyses are based on the crystal structure of subtilisin Carlsberg (PDB code 1YU6) obtained from the RCSB Protein Data Bank. YASARA Structure (Krieger et al., 2004) and VMD (Humphrey et al., 1996) were used for energy minimization and visualization. Amino acid numeration in this work is according to the amino acid order of the mature subtilisin Carlsberg form, and all amino acids starting from position 56 are shifted by one position because the PDB entry lacks position 57.

The crystal structure of subtilisin Carlsberg (PDB code 1YU6) was obtained from the RCSB Protein Data Bank and was used for *in silico* generation of energy-minimized (Krieger et al., 2004) subtilisin Carlsberg variants. For each substitution, the energetically most favorable rotamer was chosen. Energy-optimized systems were solvated using TIP3P (Miyamoto and Kollman, 1992) water molecules in a box with 15-Å distance from the protease. Three simulation annealing cycles from 298 to 5 K were performed on the second tetrahedral intermediate of subtilisin Carlsberg (*L216/M221*), parent T58A/L216W/*M221*, and variants M4 (T58A/W216M/*M221*) and M6 (T58A/W216L/M221C) using YASARA 11.9.8, the Force field AMBER03 (Duan et al., 2003), and GAFF (Wang et al., 2004). The additional bond/angle/dihedral/improper parameters and partial charges for the tetrahedral intermediate were generated following the AM1BCC AutoSMILES procedure (Jakalian et al., 2002) and are reported in the supplementary material (Tables S2–S5).

Based on a model of subtilisin Carlsberg M4 (T58A/W216M) and M6 (T58A/W216L/M221C), the solvent accessibility was determined using the Discovery Studio 3.1 Visualizer software (Accelrys, Inc., San

Diego, CA, USA). A residue was classified as 'exposed' if the solvent accessibility exceeded 25% or as 'buried' if the solvent accessibility was <10% of the maximum solvent accessibility.

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