



Variants of the industrially relevant protease KP-43 with suppressed activity under alkaline conditions developed using expanded genetic codes

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ABSTRACT

In the present study, we attempted to control the pH profile of the catalytic activity of the industrially relevant alkaline protease KP-43, by incorporating 3-nitro-L-tyrosine and 3-chloro-L-tyrosine at and near the catalytic site. Thirty KP-43 variants containing these non-natural amino acids at the specific positions were synthesized in *Escherichia coli* host cells with expanded genetic codes. The variant with 3-nitrotyrosine at position 205, near the substrate binding site, retained its catalytic activity at the neutral pH and showed a 60% activity reduction at pH 10.5. This reduction in the alkaline domain is desirable for enhancing the stability of the enzyme in the liquid laundry detergent, whereas the wild-type molecule showed a 20% increase in response to the same pH shift. The engineered pH dependency of the activity of the variant was ascribed partly to a lowered substrate affinity under the alkaline conditions, in which the incorporated 3-nitrotyrosine was probably charged negatively due to the phenolic pK_a lower than that of tyrosine.

1. Introduction

Proteases are used in various industries, and constitute an important ingredient in the laundry detergent [1–3]. Stains made of proteins, involving milk, egg white, the dirt from human bodies and others, are easily removed from fabrics, after they are digested by the enzymes. The undesirable self-digestion of proteases takes place in the liquid laundry detergent, as they are included in the active form, as opposed to the naturally-occurring proteases being synthesized in the pre-matured form. Thus, including the enzymes in liquid detergent raises a serious problem in terms of their stability during a storage period [4]. Various approaches have been taken to address this issue. For example, boronic acid derivatives are used as a stabilizer to prevent the self-digestion [5], and more stable proteases resisting the digestion have been developed through protein engineering [6,7]. In the present study, we took a new approach and applied genetic code expansion to introduce chemical groups not found in the standard amino acids into a protease. Since liquid detergents are commonly weakly alkaline, proteases are kept under this condition during storage, and then exposed to the neutral pH, when the detergent is applied to washing and thousand-

times diluted in water. We conceived that some type of non-natural amino acids would be useful for controlling the pH profile of protease activity, when they are incorporated in or near the active site of the enzyme.

2. Materials and methods

2.1. Amino acids, bacterial strains, and plasmids

3-Nitro-L-tyrosine and 3-chloro-L-tyrosine were purchased from Sigma-Aldrich. The gene encoding KP-43 was cloned from the genome of *Bacillus* sp. KSM-KP43 (FERM BP-6532). For expression of KP-43 gene, *Escherichia coli* RFzero-iy [8] or *Bacillus subtilis* 168 was used as a host strain, and Plasmid pET26b-IYN3 [8], or pHY300PLK (Takara) was used for expression vector, respectively. *Escherichia coli* RFzero-iy was used for incorporation of 3-nitro-L-tyrosine or 3-chloro-L-tyrosine into KP-43.

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2.2. Protein preparation

The transformants of *Escherichia coli* RFzero-iy for the preparation of KP-43 variants with 3-nitro-L-tyrosine or 3-chloro-L-tyrosine were grown in LB medium (0.5% yeast extract, 1% tryptone, and 0.5% NaCl) supplemented with kanamycin (30 mg/L) and 3-nitro-L-tyrosine or 3-chloro-L-tyrosine (0.2 g/L) at 37 °C for 9 h. After addition of 2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), cells were fermented at 25 °C for 24 h. The transformant of *B. subtilis* 168 for the preparation of wild type KP-43 or F205Y variant was grown on 2xL-Mal medium (1% yeast extract, 2% tryptone peptone, 1% NaCl, 7.5% maltose hydrate, and 7.5 μ g/mL MnSO₄) supplemented with tetracycline (15 mg/L) at 30 °C for 3 days. Cell suspension was centrifuged at 10,000g for 20 min at 4 °C and proteins were concentrated using Vivaspin 5000 MWCO (Sartorius). CaCl₂ was added to a final concentration of 2 mM.

2.3. Enzymatic assay

Protease activity was measured modified from [9]. The recombinant protein of each variant or wild type KP-43 was used as a crude preparation. The assay mixture contained 50 mM phosphate buffer (pH 7.0, pH 8.5, and pH 10.5) and 1 mM Glu-Ala-Ala-Pro-Leu-pNA (AAPL; Peptide Institute). This synthetic peptide was dissolved in dimethylsulfoxide. The reaction was carried out by adding a suitably diluted solution of enzyme (0.005 mL) to 0.195 mL assay mixture. The rate of the substrate hydrolysis was determined by measuring the release of *p*-nitroaniline at 405 nm at 40 °C. One unit of protease activity was defined as the amount of enzyme needed to release 1 μ mol of *p*-nitroaniline per minute at 40 °C. K_m and V_{max} were determined with AAPL over the concentration range from 0.25 to 2.0 mM by Lineweaver–Burk plot method.

3. Results and discussion

3.1. Screening of tyrosine and phenylalanine positions for the effective replacement with non-natural amino acids

In our attempt in engineering the industrially-relevant KP-43 alkaline protease [10,11], tyrosine derivatives with altered pK_a for the phenolic hydroxyl group were incorporated into KP-43 in place of phenylalanine and tyrosine residues. 3-Nitro-L-tyrosine and 3-chloro-L-tyrosine exhibit significantly lower pK_a for the hydroxyl group (7.23 and 8.48, respectively) than that of L-tyrosine (10.05). Thus, these tyrosine derivatives are electrically charged under weak alkaline conditions in which L-tyrosine stays in the neutral form, and potentially exert adverse effects on the activity of the enzyme, when they substitute for tyrosine residues in or near the active site. Although histidine also changes its electrostatic state near the neutral pH, the replacement of tyrosine with histidine would make a greater structural change and the effect could be destructive.

We used *Escherichia coli* RFzero-iy cells for incorporating these non-natural amino acids. This *E. coli* strain has been modified to lack the endogenous factor terminating translation at the UAG stop codon, instead expressing the heterologous pair of tRNA and tyrosyl-tRNA synthetase that translates UAG to 3-nitro-L-tyrosine or 3-chloro-L-tyrosine in accordance of the tyrosine derivative supplemented in growth media [8,12]. Thus, the protein products of the KP-43 mutant genes containing in-frame UAG codons exclusively included these derivatives at the UAG positions.

KP-43 consists of two domains: the N-terminal catalytic domain and the C-terminal β -barrel, as shown in a crystal structure [13]. 3-Nitro- and 3-chloro-L-tyrosines were substituted for all 10 of the tyrosines in the catalytic domain and five phenylalanines in the same domain, selected on the basis of the distance from the active site (Fig. 1). We conducted a quick screening of the 15 resulting variants for an activity reduction at higher pH (Supplementary Figs. 1 and 2).

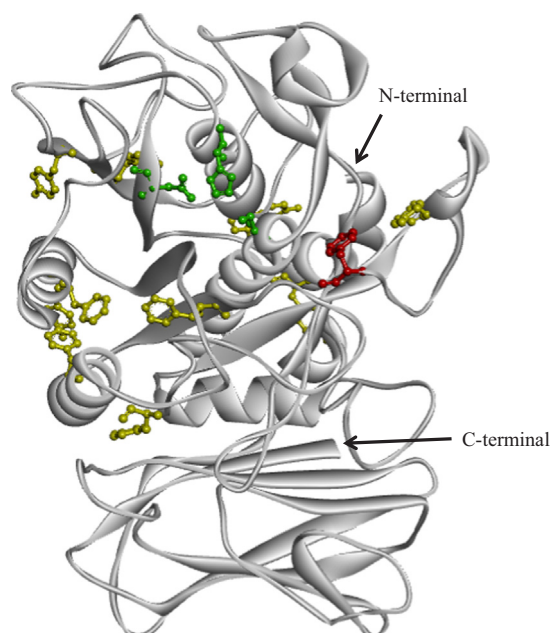


Fig. 1. The tyrosine and phenylalanine residues selected for nitration and chlorination are mapped in the crystal structure of KP-43 (PDB ID: 1WMD [13]). The residues involved in the catalytic triads are represented by the sticks and balls in green. The tyrosine and phenylalanines subjected to replacement are shown in yellow, with F205 being in red.

Among these attempted variants, the variants with 3-chloro-L-tyrosine in place of phenylalanine at positions 94 (F94) and tyrosine at position 248 (Y248) and those with 3-nitro-L-tyrosine in place of F94 and F158 were not expressed or showed no activity. Then, the catalytic activity was compared between at pH8.5 and a higher pH (pH10.5 and 11.0 for the chlorinated and nitrated variants, respectively), and it was found that the wild-type KP-43 and most of the variants hardly changed the activity level in response to the pH shift. By contrast, the three variants with 3-nitro-L-tyrosine at position 205 (F205NiY), F240NiY, and 3-chloro-L-tyrosine (F205ClY) exhibited a 40% or more reduction in the activity level at the higher pH.

3.2. Characterization of the nitrated and chlorinated KP-43 variants

The pH profile over a range from pH 7.0 to 10.5 was examined for the F205NiY and F240NiY variants, revealing that the F205NiY variant exhibited a more significant decrease in the activity under the alkali conditions (Fig. 2A). We then focused further experiments on the impact of the modifications at position 205, which is located near the substrate binding site, and examined pH profile over a range from pH 5.5 to 10.5 (Fig. 2B). The activities of the wild-type KP-43 and the F205Y variant showed similar profiles over this pH range, with a nearly steady increase in the alkaline domain. By contrast, the F205NiY and F205ClY variants showed the peak of the activity around the neutral pH, with a steady decrease in the alkaline domain. This decrease was steeper for the nitrated enzyme than the other; the activity of F205NiY was as low as 40%, relative to that of the wild-type molecule. These pH profiles of the activities of the four KP-43 molecules seemingly reflected the difference in pK_a .

Finally, we determined the kinetic parameters of the wild-type KP-43, the F205Y variant, and the variants nitrated and chlorinated on Y205 at pH 7.0, 8.5, and 10.5. The K_m value is nearly equal among these molecules at the neutral pH, although the modifications on Y205 appeared to slightly improve the substrate binding (Fig. 3A). Along the pH shift to the alkaline domain, the K_m value hardly changed with the wild-type molecule, and slightly increased with the F205Y variant. By contrast, the nitration and chlorination each caused a nearly 3-fold

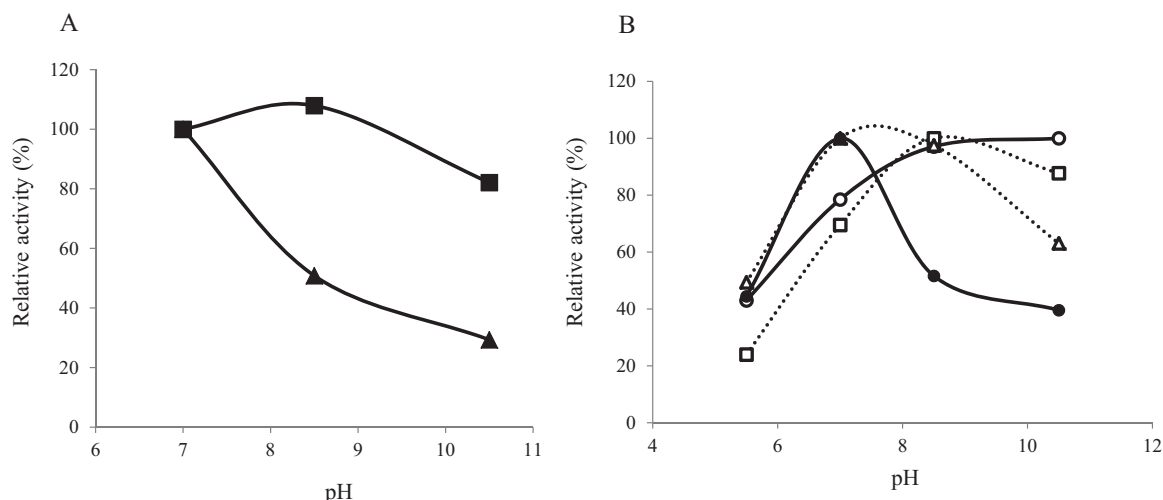


Fig. 2. (A) The relative activities to digest AAPL at pH 7.0, 8.5 and 10.5 were determined for the F205NiY variant (filled triangle) and the F240NiY variant (filled square). (B) The relative activities to digest AAPL at pH 5.5, 7.0, 8.5 and 10.5 were determined for the wild type KP-43 (open circle; solid line), the F205Y variant (open square; dotted line), the F205ClY variant (open triangle; dotted line) and the F205NiY variant (closed circle; solid line).

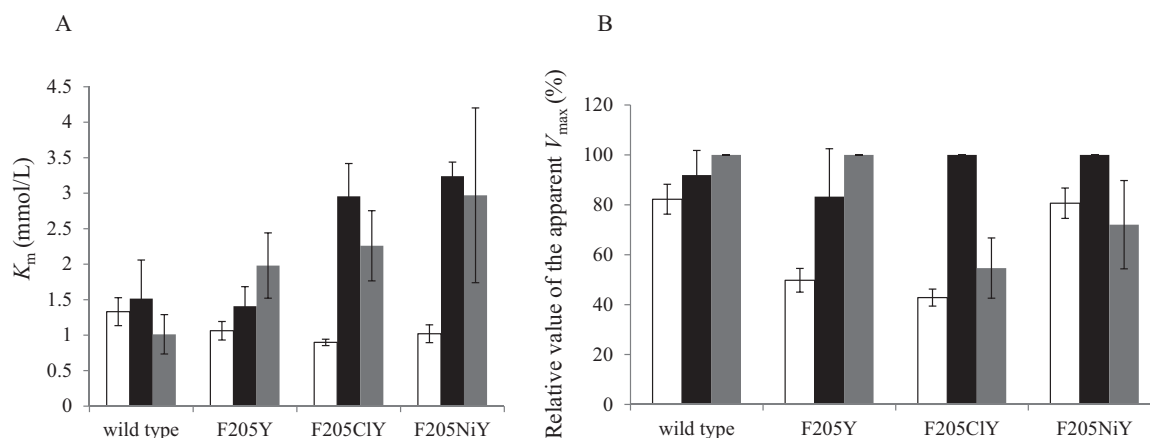


Fig. 3. The K_m values (A) and the relative V_{max} values (B) at pH 7.0 (white bar), pH 8.5 (black bar), and pH 10.5 (gray bar) were determined for the wild-type KP-43, and the indicated variants. The data were obtained from three independent experiments.

increase in K_m at pH 8.5 and 10.5. We interpreted this observation in such a way that NiY and ClY at the position near the substrate binding site were electrostatically charged under the alkaline conditions, and hampered the binding to the substrate. These nitrated and chlorinated variants showed a peak value of the relative V_{max} around pH 8.5, and exhibited similar V_{max} values at either side: pH 7.0 and 10.5 (Fig. 3B). On the other hand, the relative V_{max} steadily increased with the wild-type KP-43 and F205Y variants, making a clear contrast with the nitrated and chlorinated variants. Although the effects of these modifications on V_{max} were thus clear, they cannot immediately be explained on the basis of the different pK_a .

In the present study, we engineered KP-43 variants with an altered pH profile of the catalytic activity, and thus suggested a novel approach for solving the dilemma of the industrial protease in the liquid detergent. The direction of the change in the profile matched what was expected from the lower phenolic pK_a of the introduced tyrosine derivatives. Although the degree of activity suppression for the KP43 variants was short of making them useful in practice, our results have paved ways for designing the pH-dependent change of enzyme activities.

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Disclosure statement

There are no conflicts of interests for all authors.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.bbrep.2018.12.001](https://doi.org/10.1016/j.bbrep.2018.12.001).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.bbrep.2018.12.001](https://doi.org/10.1016/j.bbrep.2018.12.001).

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