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Short communication

A GFP-based assay for the determination of hydrolytic activity and substrate specificity of subtilisins under washing conditions

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Abstract

The serine protease subtilisin is one of the most important industrial enzymes and is mostly used as a detergent protease. The development of new proteases with improved washing performance is limited by the tedious screening of variants on stained cloths. Here, an easy and flexible green fluorescence protein (GFP) based protease assay is described, which is performed under almost authentic washing conditions. A GFP-substrate–His-tag fusion protein is hydrolyzed by subtilisin and the activity is followed by measurement of fluorescence values from the GFP released. Two detergent proteases variants (*Bacillus lentus* alkaline protease, BLAP P and BLAP X) were investigated using three model substrates (AAAAPF, AAFAAF and KHDRKD). As expected from the known substrate specificities of these proteases, AAAAPF and AAFAAF were hydrolyzed rapidly, whereas KHDRKD represents a rather stable peptide. The k_{cat}/K_M-values determined using this GFP-based assay closely matched the values using *p*-nitroanilides (pNA) of model peptides. In contrast to the use of pNA-substrates, this format is not restricted to synthetically available substrates and therefore in principle allows the screening of random peptide libraries to determine the substrate specificities of subtilisins and its variants.

Keywords: GFP; Subtilisin; Protease; Substrate specificity; Activity; Assay

1. Introduction

Subtilisins (EC 3.4.21.62) are a family of alkaline serine endoproteases secreted by a wide variety of *Bacillus* species and represent the most important commercial enzyme class used as a detergent additive and for leather processing [1,2] mostly due to their broad substrate specificity and their optimum activity at high alkaline pH. The development of new proteases with improved washing performance is limited by the screening of variants on stained cloths. This method is extremely tedious, provides only relative activities with high variations and is not compatible with high-throughput screening [3–5]. Therefore, the rapid determination of the specific activity for new mutants regarding different peptide substrates is important to understand and improve their properties. This analysis should take place under conditions that reflect the authentic washing process.

Beside several industrial applications due to their hydrolytic activity [1,6], proteases have also been important targets for drug development in a number of areas [7]. This results in a variety of protease assays [8]. Most of them are performed in solution and usually allow an easy determination of kinetic constants [9–12]. In contrast, the hydrolytic removal of proteinogenic stains occurs on the surface of the fibers, where steric effect cause additional selection criteria for improved detergent proteases [3]. This effect can influence the protease performance [13] and is not desired for most

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proteases, because there reaction takes place in solution. Furthermore, for simulating washing conditions, the assay must be performed in the presence of very high concentrations of detergent, moderate amounts of chelating reagents and are performed at alkaline pH values. This is important, because it was shown that the presence of commercial powder detergent has influence on the activity of enzymes, which can differ between variants of the same enzyme [14]. Additionally, the synthesis of chemical substrates (i.e. *p*-nitroanilide derivatized peptides) can be complex and expensive. Moreover, the use of these (activated) surrogate substrates might reveal a substrate specificity and activity, which is not identical with the true specificity and activity of the protease towards a real peptide sequence. This was demonstrated using a mutant of the subtilisin from *B. lentus* (BLAP), where the catalytic efficiency, as measured by k_{cat}/K_M (s^{-1} M^{-1}) on the artificial substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, increases more than four-fold, because k_{cat} increases and K_M decreases. In contrast, the proteolytic activity on casein is diminished compared to the non-mutant. This apparent contradiction can be explained in term of a "narrowing" of the substrate binding cleft as a result of the Ser128Gly variation, which improves the enzyme catalytic efficiency on peptides but not on peptidic substrates [15]. Therefore, engineering and screening of subtilisin mutants is an extremely complex, difficult and long process in order to obtain new proteases with improved practical performance and real commercial potential. Solutions are protease assays where the cleavage of a substrate–reporter fusion protein causes the release of the reporter protein [16–18] or protease activity detection by fluorescence resonance energy transfer (FRET) [19,20]. These fusion proteins allow the easy investigation of different substrates and surrender the need for artificial chromogenic or fluorogenic groups, which could influence the substrate specificity and activity. Additionally, they allow the influence of the prime site residues, which are generally not considered to determine the substrate specificity of subtilisins. It was shown that the prime site Tyr217Leu mutation in subtilisin Carlsberg (equivalent to position 211 in BLAP) provides twice as much cleaning performance through accelerating k_{cat} [21]. Even this variant shows the same order of P1 substrate preference with *p*-nitroanilide, other variants show differences in their preference [22], which can cause the observed differences in washing performance.

Thus, a major challenge is to construct a protease assay for peptides, which is only cleaved in the substrate sequence by the non-specific subtilisin. The reporter protein and the immobilization tag must be resistant to subtilisin, while the substrate must be accessible for subtilisin.

In the present study, a protease assay based on a green fluorescent protein (GFP)-substrate–His-tag fusion protein was developed and verified under almost authentic washing conditions. The proteases (BLAP P and BLAP X) investigated are used in commercial available detergents.

2. Materials and methods

Isopropyl thio-β-D-galactoside (IPTG) was purchased from Gerbu (Gaiberg, Germany). Primers were synthesized by Invitrogen (Karlsruhe, Germany). The Ni-NTA beads were purchased from Qiagen (Hilden, Germany), while the QuikChange Site-Directed Mutagenesis Kit was from Stratagene (San Diego, USA). 96F Maxisorp Black Microwell SH was purchased from Nalga Nunc International, Brand Products (Wertheim, Germany). The synthetic substrates (AAPF–pNA, FAAF–pNA) were obtained from Bachem (Heidelberg, Germany). Restriction enzymes were from New England Biolabs (Frankfurt am Main, Germany). pET-29a(+) was purchased from Novagen (Madison, USA). The two *Bacillus lentus* alkaline proteases (BLAP) variants and the protease-free detergent powder (Persil Color (powder/liquid) as produced and marketed in 2003) were obtained from Henkel KGaA (Düsseldorf, Germany). The commercial available Persil Color detergent powder contains a BLAP variant for the removal of proteinaceous stains. BLAP P (S3T, V4I, V199I, L211D) and BLAP X (S3T, V4I, V199I) are mutants of BLAP [23].

2.1. Expression of the GFP-substrate–His-tag

After deletion of the *Nde*I restriction site at position H77 and M78 in GFP (pIVEX-GFP, Roche, Mannheim, Germany) with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) (silent mutation sites are underlined) (5′-GCT TTT CAA GAT ACC CGG ATC ACA TGA AAC GGC ATG AC-3′ and 5′-GGC ACT CTT GAA AAA GTC ATG CCG TTT CAT GTG ATC CGG GTA TC-3′), the *GFP* gene was amplified using primers, which framed the gene with *Nde*I and *Bgl*II sites (underlined): 5′GAG GAT TTA ACA TAT GAC TAA AGG TGA AGA ACT TTT CAC TGG AGT TGT CCC-3′ and 5′-TTC GCA TGT TAG ATC TTT GGT ACA GTT CAT CCA TGC CAT GTG TAA TCC C-3′. After the *Nde*I and *Bgl*II digestion, the gene was cloned into the pET-29a(+) vector from Novagen. The substrate encoding DNA sequences were synthesized by two primers according to the method from Sparks et al. [24]. The desired substrate sequences were framed on the 5′-end by a *Bgl*II restriction site and a GPGG encoding sequence and on the 3′-site by a GGPG encoding sequence (in italics) and *Apa*I/*Xho*I restriction sites (underlined). GPGG and GGPG, respectively, were introduced as linkers, which ensure the autonomous folding of the individual elements. The peptide sequences AAAAPF (5′-GGA TTT AAA GAT CTC *GGC CCG GGT GGC* GCG GCT GCG GCA CCG TTC GGC GGT CCG-3′ and 5′-TCG CAT GTT CTC GAG GGG CCC *ACC CGG ACC GCC* GAA CGG TGC CGC AGC CGC GCC-3′) and AAFAAF (5′-GGA TTT AAA GAT CTG *GCC CGG GTG GCG* CGG CTT TCG CAG CGT TCG G-3′, 5′-TCG CAT GTT GGG CCC *ACC CGG GCC ACC* GAA CGC TGC GAA AGC CGC-3′) were chosen as sensitive substrates, while KHDRKD (5′-GGA TTT AAA GAT CTG *GCC CGG GTG GCA* AAC ACG ACC

GTA AAG ATG G-3′ and 5′-TCG CAT GTT GGG CCC *ACC CGG GCC ACC* ATC TTT ACG GTC GTG TTT GCC-3′) was chosen as a resistant substrate sequence. The synthesized fragment was digested with *Bgl*II and *Xho*I and cloned into the GFP containing pET-29a(+) vector. The resulting vector was transformed into *E. coli* BL21 cells.

In order to produce the recombinant proteins, the cells were grown in LB media with ampicillin (100 μg mL^{-1}). Cells were induced with 1 mM IPTG at an OD_{600} = 1.0 and grown overnight at 24 °C. After harvesting and cell lysis, the fusion proteins were immobilized on Ni-NTA beads (Qiagen) for 1 h at 4 °C following the manufacturers protocol. In addition, two further washing steps with enzyme-free Persil Color solution (6 g/L) were included. The immobilized fusion proteins were stored at 4 °C.

2.2. Determination of the hydrolytic activity of subtilisin

For the determination of the specific activity (fluorescence/amount of protein) an aliquot of the immobilized protein was eluted and the initial protein concentration [S_0] was determined by the bicinchoninic acid assay (KMF Laborchemie Handels GmbH, Lohmar, Germany). The Ni-NTA-beads with the immobilized fusion protein were resuspended in enzyme-free Persil Color solution to obtain a 10% (w/v) solution. Several sequential dilutions were prepared from this solution by the addition of Persil Color solution (up to 625 μL). After adding 75 μL of the subtilisin solution, the end volume was set to 750 μl. All pipetting steps were performed on ice, to prevent pre-digestion of the fusion proteins. After 20, 40 and 60 min incubation at 30 °C and 10 rpm with the Rotamix RM-1 (ELMI Ltd., Riga, Latvia) the solutions were kept on ice. The Ni-NTA beads were pelleted by centrifugation at 1000 g for 4 min at 4 °C. The supernatant was kept on ice before fluorescence measurement (Fluostar Galaxy, BMG Labtechnologies GmbH, Offenburg, Germany). Each sample was measured twice. The percentage of hydrolysis was calculated as the ratio of released GFP to the initial amount of GFP bound to the beads, which was quantified by elution with imidazole. Specificity constants (k_{cat}/K_M) were determined from Lineweaver–Burk plots.

Scheme 1. Gene III-fusion protein. GFP: green fluorescent protein; His-tag: six histidines as immobilization tag.

The kinetic determination with pNA-substrates were made in 0.1 M Tris, 10% Brij35, pH 9.0 at 30 °C and reaction progress curves were analyzed as described [22].

3. Results and discussion

In this study, a green fluorescence protein-substrate–His-tag fusion protein was used for the determination of the proteolytic activity of the detergent proteases BLAP P and BLAP X against three different model substrates (Scheme 1). GFP was chosen as reporter protein because of its robustness, especially against cleavage by proteases [25]. To establish this method, AAAAPF and AAFAAF were chosen, as they closely match commercially available synthetic *p*-nitroanilides of AAPF and FAAF. In addition, the peptide sequence KHDRKD was used as subtilisin-stable substrate motif.

First, the stability of GFP against the subtilisins BLAP P and BLAP X was verified by digestion at 30 and 50 °C at a 20-fold excess of the highest protease concentration used during the protease assay. This is more than usually used in the commercial available Persil detergent powder. As expected, no decrease of fluorescence was observed (data not shown). This is consistent with the observation, that GFP is highly stable towards proteolytic degradation, as reported for a 2-day treatment with various proteases (trypsin, chymotrypsin, papain, subtilisin, thermolysin and pancreatin) under optimal conditions and protease concentrations as great as 1 mg mL^{-1} [26]. Next, the fusion proteins with the three model peptides were investigated. It is obvious from Table 1 that both proteases showed high activity towards the motifs derived from common *p*-nitroanilide-based substrates. AAFAAF is hydrolyzed approximately 12-times (BLAP P) and 6-times (BLAP X) faster than AAAAPF. The higher preference of high alkaline subtilisins for the FAAF-substrate is in accor-

Table 1
Comparison of the hydrolytic activities of the subtilisins BLAP P and BLAP X using the GFP-fusion proteins containing specific peptide sequences and pNA, respectively, under washing conditions

	k_{cat} (s^{-1})		K_M (mM)		k_{cat}/K_m (10^3 M^{-1} s^{-1})		$(k_{cat}/K_M)_{FAAF}/(k_{cat}/K_M)_{AAPF}$	
	pNA	GFP-assay	pNA	GFP-assay	pNA	GFP-assay	pNA	GFP-assay
BLAP P								
KHDRKD		1.5		2.39		0.64		
AAFAAF	479.3	53.1	0.79	0.37	606.71	142.54	44.59	11.53
AAAAPF	34.8	9.7	2.56	0.78	13.59	12.36		
BLAP X								
KHDRKD		3.6		3.42		1.06		
AAFAAF	365.9	112.2	0.05	0.39	6903.77	291.62	23.29	6.35
AAAAPF	386.0	44.8	1.30	0.98	296.47	45.92		

dance with literature data [27,28]. The same specificity was observed for the *p*-nitroanilide-based substrates (Table 1). As expected for these substantially different substrates (GFP versus pNA-residue), the kinetic values were not identical, but they are in the same range. However, the degree of preference for the FAAF over AAPF differs. This is most probably due to the artificial chromophoric group, because it was suggested that the *p*-nitroanilide leaving group interacts with the amino acid residue 211 [22]. This difference seems to be subtle, but could play in important role for explaining the differences in washing performance. As expected, KHDRKD is only a poor substrate for both proteases and was hydrolyzed at substantially lower rates (222-fold, BLAP P; 275-fold, BLAP X).

In addition, the GFP-substrates turned out to be very stable, as a 2-months storage at 4 and −20 °C with weekly determination of protease activity gave no change in the activity data. Therefore, these substrates are very well suited for the routine determination of protease activity.

This assay has the major advantage, that a peptidic fluorophore is directly attached to the peptide sequence to be cleaved, which simulates peptidic stains much better than synthetic substrates bearing chromophores or fluorophores like *p*-nitroanilide. Most importantly, any peptide sequence (including random sequences) can be investigated under conditions, which are close to real washing conditions or for an assay on stained cloth. In principle, this protease assay can also be adapted to a high-throughput screening assay for detergent proteases, using magnetic Ni-NTA beads, or using plates with a filter bottom followed by the removal of the non-cleaved substrate through filtration. This enables the substrate profiling of proteases, thus leading to a fingerprint of a certain protease by digesting a model substrates or in combination with selection based substrate phage display [29–31]. If necessary, the cleavage site of the protease can be determined by N-terminal sequencing of the cleavage products.

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