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Short communication

Multi-site saturation by OmniChange yields a pH- and thermally improved phytase

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ABSTRACT

Directed evolution of *Yersinia mollaretii* phytase (Ymphytase) yielded an improved variant SM2P3E4 (also named M1; D52N, T77K, K139E, G187S, V298M) in our previous study. Variant M1 retained high specific activity (993 U/mg; equivalent to 93% of wild-type activity) and improved thermal resistance (T50 improved by 1.5 °C compared to wild-type at 58 °C; 20 min incubation time), making variant M1 an attractive enzyme for industrial applications. Recently, the OmniChange method was developed for multi-site saturation mutagenesis. The five sites identified in variant M1 were subjected to OmniChange saturation in order to explore whether a variant with higher activity, higher thermal resistance, and higher resistance at low pH (2–3 h incubation was performed to mimic the gastric residence time of phytase) could be identified. Screening of a small library of 1100 clones, covering <0.004% of the theoretical sequence space of 3.35×10^7 variants, yielded a Ymphytase variant with 32% improved residual activity (58 °C for 20 min), 2 °C increased apparent melting temperature (T_m), and 2-fold higher pH stability (pH 2.8; 3 h incubation time) when compared to the wild-type Ymphytase. Compared to the M1 variant, the pH stability (pH 2.8; 3 h incubation time) was improved by 3-fold, and thermal resistance as well as activity was improved slightly (residual activity: 32% compared to 20%; apparent T_m: 2 °C compared to 1.5 °C; activity difference <4%).

1. Introduction

Directed evolution has been applied successfully to improve enzyme properties and to explore structure–function relationships (Bershtein and Tawfik, 2008; Shivange et al., 2009). In most directed evolution campaigns, substitutions were introduced with a rate of one to two amino acid substitutions per round of directed evolution (Tracewell and Arnold, 2009). Rational and semi-rational protein engineering has successfully been employed to improve "localized" properties such as activity or enantioselectivity (Reetz et al., 2010). The latter was achieved by iterative site-directed or site saturation mutagenesis at positions identified through structural analysis or sequence alignments (Bornscheuer and Pohl, 2001; Nestl et al., 2011). In addition, iterative rounds of site-directed and site-saturation mutagenesis in the substrate binding pocket, named CASTing, proved to be an efficient approach in improving enzyme properties (Reetz et al., 2005). Since selected positions for mutagenesis are often in close proximity to each other, it is likely that side chains influence each other so that synergistic substitutions that might require the simultaneous exchanges of two or more amino acids are missed. For instance, Schmitzer et al. (2004) reported that generation of 1536 variants by mutating four positions simultaneously yielded in three out of 1536 variants with "novel functional active site patterns".

Phytases catalyze the release of inorganic phosphate by hydrolysis of phytate that makes 50–80% of the phosphorous storage in cereal grains, oilseeds, and legumes. Phytate is hardly digestible by monogastric animals resulting in reduced bioavailability and phosphate excretion causing environmental pollution. Supplementation of phytase in animal feed reduces phosphate excretion and improves hydrolysis of phytate. For industrial applications, phytases should have a high activity and a high thermostability which is required in the feed pelleting process (60–80 °C). Recently, we have reported a highly active phytase from *Yersinia mollaretii* (Ymphytase; specific activity 1073 U/mg) and directed evolution of Ymphytase (epPCR (~6800 clones) SeSaM (~2350 clones)) yielded a variant M1 (previously named SM2P3E4) with five substitutions (D52N, T77K, K139E, G187S, and V298M) and improved thermal resistance (Shivange et al., 2011).

Multiple site saturation mutagenesis of five positions simultaneously represents the upper limits coverable with high throughput screening technologies (3.2×10^6 different protein

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variants; 3.4×10^7 different codon variants). OmniChange is a multi-site saturation mutagenesis methods, developed to saturate up to five sites simultaneously allowing to explore novel combination of substitutions that may interact with each other and/or exhibit epistatic effects (i.e. non-additive interactions between mutations that affect enzyme property) (Dennig et al., 2011). The latter types of substitutions will likely not be identified in iterative saturation mutagenesis of single sites. In the present manuscript, we report an Ymphytase variant with higher thermal resistance and higher resistance at low pH, obtained by screening a focused OmniChange mutant library (5 simultaneously saturated positions). The improved variant was obtained with "limited" screening efforts (1100 clones; <0.004% of theoretical sequence space). The obtained results were compared to Ymphytase variant (M1) which was identified from a directed evolution campaign with randomly introduced mutations (2 rounds; one epPCR and one SeSaM; and screening of 9150 clones).

2. Materials and methods

All oligonucleotides used in this study are summarized in Supplementary Table S1 and the OmniChange mutant library was generated by assembling gene fragments (A–D) with vector fragments as previously described (Dennig et al., 2011) (see Supplementary material).

2.1. Screening of OmniChange mutant libraries

A high-throughput prescreening was performed as described previously (Shivange et al., 2011) using 384-well microtiter plates followed by a screening in 96-well microtiter plates using 4-methylumbelliferyl phosphate substrate (see Supplementary material).

2.2. Characterization of wild-type Ymphytase and variant Omni1

Ymphytase wild-type and mutant Omni1 were purified, activity and thermal resistance were determined as described previously (Shivange et al., 2011). The pH profile and pH stability of wild-type and Omni1 Ymphytase variant were determined at 37 °C using four different buffers: 0.25 M glycine–HCl buffer for pH 2.0–3.2, 0.25 M sodium acetate buffer for pH 3.6–5.6, 0.25 M imidazole–HCl buffer for pH 6.0–7.0 and 0.25 M Tris–HCl for pH 7.4–9.0. Purified enzymes were diluted in specified buffers to 40 ng protein per milliliter and assays were carried out with 1 mM phytate with corresponding pH using AMol assay (Shivange et al., 2011). The pH stability was determined by incubating Ymphytases at 37 °C for 3 h and residual activity was measured using AMol assay. Non pH treated enzyme activity was considered as 100% activity.

3. Results and discussion

In order to determine whether any of the mutations in the previously described M1 variant (Shivange et al., 2011) are individually responsible for the improved properties, each mutation (D52N, T77K, K139E, G187S, or V298M) was introduced into the wild-type Ymphytase to generate each single mutein. The position T77K improved thermal resistance without affecting activity. All other positions had individually no beneficial influence on thermal resistance or activity (Supplementary material Fig. S1).

Mutational spectrum of the OmniChange mutant library was assessed by sequencing 30 picked clones (18 active clones and 12 inactive clones: Table 1). Out of 30 sequenced clones, only 1 variant possessed a secondary transition mutation outside of the targeted position (G151S (GGT → AGT)). The asterisk (*) in Table 1 indicates

Table 1
Amino acid substitution spectra of the OmniChange mutant library. OmniChange variants were ranked based on their activity at room temperature (++ = 70–105%; + = 20–70%; – = inactive; compared to wild-type Ymphytase).

Rank	Variant	Substitution Position					SS	Activity
		D52	T77	K139	G187	V298		
1	P17G2	S	K	D	N	G		++
2	P18F11	D	E	L	S	L		++
3	P18F2	D	R	L	L	M		++
4	P18G6	D	G	G	S	M	T299S[#]	++
5	P18C2	L	R	G	S	W		++
6	P17G7	H	K	G	S	I		++
7	P17G5	D	V	D	E	L		++
8	**Omni1** P17D8[§]	E	T	T	S	F		++
9	P18G1	D	M	R	K	M	G151S	++
10	P18B2	D	S	N	V	A		++
11	P17C5	N	V	M	G	V		++
12	P18D5	D	K	I	T	L		++
13	P18C9	V	S	T	Y	S		+
14	P18A4	H	T	A	F	I		+
15	P17A8	E	R	K	K	V	D137Y[#]	+
16	P18F5	D	A	A	L	R		+
17	P17B6	P	S	R	A	Q	P59A[#]	+
18	P17C3	C	V	Q	L	I		+
19	P17B2	M	*	R	E	P	A149S[#]	-
20	P18B10	D	P	S	Y	N		-
21	P17F6	G	M	F	Y	C		-
22	P17A4	E	K	P	R	A		-
23	P18C12	D	C	R	D	G		-
24	P17C6	E	H	P	*	*		-
25	P17E12	A	P	K	T	F		-
26	P18A8	L	*	S	V	P		-
27	P18B6	D	P	S	*	P		-
28	P18C4	V	C	*	P	P	A149V[#]	-
29	P18E1	D	I	N	M	Δ	I297F[#]	-
30	P17D1	F	Q	C	*	P		-
Substitutions property in the active clones		56% negetively charged	33% positively charged	Mixed	61% Polar	83% Hydrophobic		

SS, secondary substitution; activity measured in cell lysate (70–100%, ++; 20–50%, +; inactive, –), clones are ranked by decrease in thermostability (1–30); amino acid color code: charged positive (H, K and R; blue); charged negative (D and E; pink); neutral (C, P, S, T, N and Q; green); aliphatic (G, A, V, L, I and M; white); aromatic (F, W and Y; magenta); proline and glycine (brown); cysteine (yellow); and stop codon (red).
[§]Omni1variant.
[*]Stop codon.
[#]Primer synthesis error.

seven clones which contained a stop codon at NNK saturated positions (NNK: 32 different codons encoding all 20 amino acids and a stop codon). Further mutations occurring within the sequence of ordered primers are marked with "#" character. Mutations of (#)-type were not observed in previously reported OmniChange mutant libraries (Dennig et al., 2011). Mutations can therefore likely be attributed to employed primers despite that two of six mutations are in close proximity to the NNK-codon (see Table 1).

Approximately, 1100 OmniChange muteins (variants arising from the respective parent gene by at least one amino acid substitution) were prescreened using a previously reported high throughput screening method using 4-methylumbelliferryl phosphate (4-MUP) in 384-well microtiter plate format (Shivange et al., 2011). Out of 1100, about 227 clones showed >80% activity compared to wild-type Ymphytase. These 227 clones were expressed and screened for thermostability in 96-well microtiter plate using 4-MUP assay. A total of 19 variants were selected and subsequently expressed in test tube. The Ymphytase variant Omni1 (P17D8; see