Research Article

Directed evolution of a *Bacillus* chitinase

Chomphunuch Songsiriritthigul[1], Putarika Pesatcha[2], Vincent G.H. Eijsink[3] and Montarop Yamabhai[2]

[1] National Synchrotron Research Center, Nakhon Ratchasima, Thailand
[2] School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand
[3] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

Chitinases have potential in various industrial applications including bioconversion of chitin waste from crustacean shells into chito-oligosaccharide-based value-added products. For industrial applications, obtaining suitable chitinases for efficient bioconversion processes will be beneficial. In this study, we established a straightforward directed evolution method for creating chitinase variants with improved properties. A library of mutant chitinases was constructed by error-prone PCR and DNA shuffling of two highly similar (99% identical) chitinase genes from *Bacillus licheniformis*. Activity screening was done in two steps: first, activity towards colloidal chitin was screened for on culturing plates (halo formation). This was followed by screening activity towards the chitotriose analogue *p*-nitrophenyl-β-1,4-*N*,*N*'-diacetyl-chitobiose at various pH in microtiter plates. From a medium-throughput screening (517 colonies), we were able to isolate one mutant that demonstrated improved catalytic activity. When using *p*-nitrophenyl-β-1,4-*N*,*N*'-diacetyl-chitobiose as substrate, the overall catalytic efficiency, k_{cat}/K_m of the improved chitinase was 2.7- and 2.3-fold higher than the average k_{cat}/K_m of wild types at pH 3.0 and 6.0, respectively. The mutant contained four residues that did not occur in either of the wild types. The approach presented here can easily be adopted for directed evolution of suitable chitinases for various applications.

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1 Introduction

Chitinases (EC 3.2.1.14) belong to glycosyl hydrolase (GH) family 18 and 19 [1]. They comprise a group of enzymes that hydrolyze chitin by cleaving its β-1,4 *N*-glycosidic bond. Endochitinases cleave randomly at internal sites of chitin, generating soluble low mass multimers of GlcNAc such as chitotetraose, chitotriose, and chitobiose [2, 3]. Chitinases have potential in various industrial applications, including degradation of chitin into chitooligosaccharides of different lengths, which are useful in food and pharmaceutical applications [4, 5]. In Thailand, crustaceans (prawn, crab, shrimp, and lobster) constitute one of the main agricultural products and they represent an abundant source of chitinous co-products. Traditional methods for chitin waste disposal include landfill, incineration, and ocean dumping. These methods are costly, inefficient, and, most importantly, harmful to the environment. An alternative method for the exploitation of chitin-rich co-products is using enzymes to convert the chitin into useful products [6]. The process of extracting chitin from crustaceans requires the use of HCl to extract minerals and NaOH to degrade proteins [7]. Thus, obtaining chitinases that are active under acidic or alkaline conditions, in addition to being stable and thermo-tolerant, will be beneficial for the exploitation of these enzymes in industrial applications.

Recent advances in molecular biology and genetic engineering have led to rapid methods for im-

Correspondence: Professor Montarop Yamabhai, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima 30000, Thailand
E-mail: montarop@sut.ac.th
Fax: +66-44-224150

Abbreviation: GlcNAc, N-acetylglucosamine; **GH**, glycosyl hydrolase; **p-NP-(GlcNAc)2**, p-nitrophenyl-beta-1,4-N,N'-diacetyl-chitobiose; **ChBD**, Chitin binding domain; **FnIII**, fibronectin type III domain

proving protein and enzyme properties [8]. One current technology, termed directed evolution, mimics the natural selection process, where genes are evolved through recursive rounds of mutation, recombination, and selection [9]. One of the most efficient methods used to direct the evolution of proteins is DNA shuffling [10]. This method allows a much larger spectrum of diversity to be generated than by natural recombination or mutational mechanisms, because two or more homologs from multiple species in different ratios are used for recombination [11, 12]. Successful applications of DNA shuffling and other directed evolution techniques for the improvement or alteration of proteins and enzymes for various purposes have been reported [9, 13]. Application of this technology for the improvement of chitinases for bioremediation and bioconversion of chitin waste is attractive. One of the key issues is to develop methods that permit high-throughput screening of chitinase properties at reasonable costs.

In this study, we have established a simple screening method that permits relatively straightforward directed evolution of chitinases. The method uses FLAG vectors that direct secretion of the chitinase. For testing the method we used a *Bacillus* chitinase for which we previously demonstrated efficient expression and secretion in *E. coli* [14]. Starting off with two almost identical chitinase genes (*chiA*) from *Bacillus licheniformis* (strains DSM13 and DSM8785), a mutant library was constructed by a combination of error-prone PCR and DNA shuffling. To demonstrate the feasibility of the method, we performed a medium-throughput screening, which yielded one mutant with improved catalytic activity.

2 Materials and methods

2.1 Bacterial strains and growth conditions

B. licheniformis DSM13/ATCC 14850 [15], and DSM8785, which were used as the templates for chitinase genes, were obtained from American Type Culture Collection and Prof. Dietmar Haltrich, respectively. The bacteria were grown and kept in M1 medium at 30°C. *E. coli* strains TOP10 were used as a host for library construction and protein expression. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C with shaking.

2.2 Construction of mutant chitinase library

2.2.1 Error-prone PCR

Error-prone PCR was preformed according to a published protocol [16]. Recombinant chitinase genes from *B. licheniformis* strain DSM13 and DSM8785 (*chiA13*, GenBank accession number AAU21943, & *chiA8785*, GenBank accession number FJ465148, respectively) [14] were used as the templates to amplify the chitinase genes under conditions promoting misincorporations of nucleotides. The PCR reaction mixtures consisted of 10 ng DNA template, dNTPs (New England BioLabs) at concentrations of 0.2 mM dATP, 0.2 mM dGTP, 1.0 mM dCTP, 1.0 mM dTTP, 2.9 mM $MgCl_2$, 0.15 mM $MnCl_2$, 5 U Taq DNA polymerase (New England BioLabs), 1× Taq DNA polymerase buffer, and 0.3 μM of each of the primers: chiHind3 (5′-CTG TGC AAG CTT TTG TCA TGT TGC TGA GCT TGT CAT TTG-3′) and chiXhoI (5′-CTG TGC CTC GAG TCC ATT TGA CTT TCT GTT ATT CGC AGC CTC-3′). The reactions were run in a PCT-200 Peltier Thermo cycler (MJ Research). Preheating at 95°C for 2 min was followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 50 s, and extension at 72°C for 2 min, with final extension at 72°C for 10 min. The products (designated Er13 and Er8785) were verified for correct size on an agarose gel, and purified using the QIA PCR purification Kit (Qiagen).

2.2.2 DNA shuffling

The second part of evolution involved the shuffling of the mutant genes from the first step according to Joern J.M [17]: approximately 5 μg of each PCR product (Er13 and Er8785) were digested with 0.1 U DNaseI (Fermentas) in 50 mM Tris-HCl pH 7.4, 10 mM $MnCl_2$ at 15°C for 7 min. The reaction was terminated by addition of ice-cooled 0.5 M EDTA pH 8.0. The DNA fragments sizes between 25 and 250 bp (designated DNaseEr13 and DNaseEr8785) were isolated from the polyacrylamide gel, following the Crush and Soak method [18]. Fragment reassembly and PCR amplification of reassembled products were done according to previously published protocol [19]. The purified SH13/8785 products were then cloned into the pFLAG-CTS vector and the constructs were transformed into *E. coli* TOP10 by electroporation as described by Sambrook and Russell [18], using an Electroporator 2510 (Eppendorf, Germany). The cloning of the library of mutant chitinases was done such that the mature enzymes were fused with *E. coli* OmpA signal peptide of the pFLAG-CTS (sigma) expression vector, as described previously [14].

2.3 Medium-throughout screening of mutant chitinases

2.3.1 First round screening on plate

E. coli transformants carrying a library of SH13/8785 genes were grown in parallel on two types of LB agar plates containing 100 μg/mL of ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The screening plate contained 0.2% colloidal chitin, whereas the master plate did not. After incubating the plates at 37°C for 18 h, 0.2% Congo Red (Sigma) was poured onto the screening plate. Colonies that produced clear zones (positive clone) were selected for a second round screening. *E. coli* carrying pFLAG-CTS constructs with the wild-type genes (pFchi13 and pFchi8785) and the pFLAG-CTS vector, were used as positive and negative controls, respectively. Chitinase-positive clones were picked from the master plate to produce 15% glycerol stocks, which were kept at –20°C for further analysis.

2.3.2 Second round screening in microtiter plate

The positive colonies from the first round of screening, as well as the two positive controls and the negative control were inoculated into 0.5 mL LB medium containing 100 μg/mL ampicillin in 96-deep well plates (Eppendorf, Germany), and incubated at 37°C with shaking. After 6 h of incubation, enzyme production was induced by adding 0.5 mL LB medium containing 100 μg/mL ampicillin and 2 mM IPTG (final concentration was 1 mM). After another 16 h of incubation at 37°C with shaking, the cultures were chilled in an icebox for 5 min and then centrifuged at 4000 rpm for 20 min at 4°C to collect the cells. The cells were resuspended in 50 μL cold (4°C) spheroplast buffer (100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 μg/mL phenylmethylsulfonyl fluoride, PMSF). After incubation for 5 min on ice, bacterial cells were collected by centrifugation at 4000 rpm at 4°C for 20 min and re-suspended in 25 μL cold sterile water supplemented with a protease inhibitor (PMSF, from Sigma). The cell suspension was incubated for 45 s on ice and subsequently mixed with 1.67 μL 20 mM $MgCl_2$. This procedure yielded a periplasmic extract of nearly 60 μL that was collected by centrifugation at 4000 rpm at 4°C for 20 min. To measure catalytic activity in microtiter plates (Nunc, Denmark), 10 μL periplasmic fraction was added to 100 μL 0.18 mM *p*-NP-$(GlcNAc)_2$ in 50 mM buffer (glycine-HCl, pH 3.0; sodium phosphate, pH 6.0, and glycine-NaOH, pH 10.0) and the mixture was incubated at 37°C for 1 h. The enzyme reaction was terminated by adding 10 μL 1 N NaOH, and the amount of *p*-nitrophenyl released from p-NP-$(GlcNAc)_2$ was measured by recording the absorbance at 405 nm. Control measurements showed that the wild-type enzymes converted less than 20% of the substrate under these conditions.

2.4 Chitinase expression and purification

To avoid artifacts, the screening and selection procedure was done using ChiA without any affinity tag. To purify ChiA variants, the wild-type and 128mt genes were fused to a C-terminal His-tag and inserted into the pFLAG-CTS expression vector by PCR cloning using chiHind3 and chi6HXho (5′-GCA CAG CTC GAG TCA GTG GTG GTG GTG GTG GTG TTC GCA GCC TCC GAT CAG CCG CC-3′) as primers. The reverse primer contains a hexahistidine tag for affinity purification of the recombinant ChiA. The PCR thermal profile consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 45 s, 56°C for 55 s, and 72°C for 2 min, followed by final extension step at 72°C for 10 min. The constructs were transformed into *E. coli* Top10. Transformants containing the correct constructs were cultured in LB medium overnight before transferring into 500 mL LB medium containing 100 μg/mL ampicillin. After culturing at 37°C until OD_{600} reached 1.0, cells were induced with IPTG at a final concentration of 1 mM, followed by further culturing at 28°C for 4 h. The cells were then harvested by centrifugation at 8000 rpm at 4°C for 20 min. Crude enzyme was prepared from bacterial cells by resuspending in 1–2 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0), followed by sonication (Ultrasonic Processor; 60 amplitude, pulses of 6 s, for 2 min) on ice. The cell debris was then spun down at 10 000 × *g* and the supernatant was collected as the cell lysate. The supernatant containing soluble chitinase was used for further purification using Ni-NTA resins according to Qiagen's protocol. The resin was washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl and 40 mM imidazole, pH 8.0), and eluted with elution buffer containing 250 mM imidazole. Fractions containing eluted chitinase were pooled and concentrated using Vivaspin membrane concentrators (MW cut-off 30 000). Concentrated enzyme solutions were divided into aliquots and kept at –20°C in sodium phosphate buffer, pH 6.0 containing 15% glycerol for further analysis.

2.5 Chitinase assay

To determine kinetic parameters, enzyme reactions were set up consisting of 0.4 μg pure enzyme and 0.008–0.7 mM of the substrate, *p*-NP-$(GlcNAc)_2$, in 100 mM sodium phosphate buffer (pH 6.0), with a

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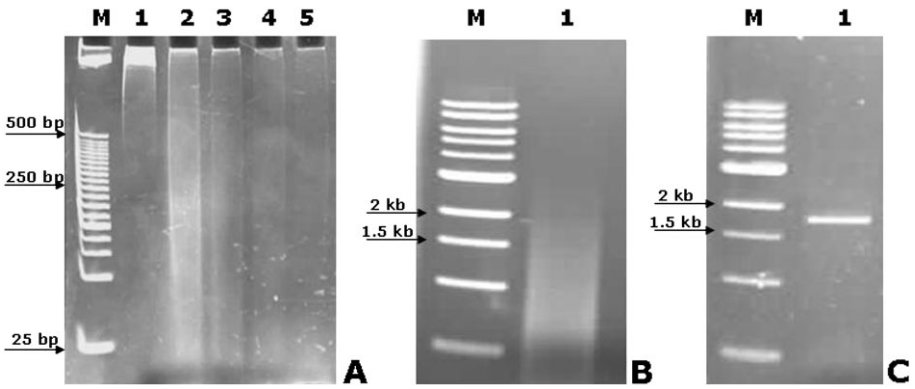

Figure 1. DNA shuffling of ChiA. Error-prone PCR products of ChiA genes from *B. licheniformis* strain DSM13 and DSM8785 were shuffled together. (A) The results of DNaseI digestion at various concentrations; M, 25-bp DNA ladder; 1, DNaseI 0.0 U/μg; 2, DNaseI 0.02 U/μg; 3, DNaseI 0.04 U/μg; 4, DNaseI 0.07 U/μg; and 5, DNaseI 0.5 U/μg. (B) Products after reassembling using Pfu DNA polymerase. (C) The shuffled ChiA genes (1.7-kb band) that have been amplified by PCR in the presence of specific primers.

total volume of 100 μL, unless otherwise stated. After incubation at 37°C for 30 min, the reaction was terminated by adding 10 μL 1 N NaOH, and the amount of *p*-nitrophenol released from *p*-NP-$(GlcNAc)_2$ was measured by recording absorbance at 405 nm, using a standard curve for pNP. Before determining the kinetic parameters, it was established that product formation was linear over time under the conditions of this assay. Reaction conditions were such that the amount of substrate converted was below 20% of the total amount of substrate added, at all substrate concentrations used in this study. The values of the kinetic constants were calculated from the Michaelis-Menten plot using a non-linear curve fitting method (GraphPad Prism 5).

3 Results

3.1 Construction of a library of mutant chitinases

Since *chiA* genes from *B. licheniformis* DSM 13 and DSM 8785 were highly similar with only five base pairs and three amino acid differences, we decided to create more diversity in the population of the genes by error-prone PCR before DNA shuffling. The *chiA* genes [14] were first cloned into the pFLAG-CTS vector and used as templates for error-prone PCR, using low fidelity buffer conditions. DNA sequence analysis of a population of the PCR products revealed that the mutation rate was approximately 0.3% (which would correspond to about five point mutations for the *chiA* gene). The mutations comprised all types of substitutions (*i.e.*, A→G, G→A, T→C, and C→T) and were distributed over the entire gene. Testing of chitinase activity in ten randomly picked clones yielded both variants where the catalytic activity was completely lost and variants that were still active. For example, one variant without chitinase activity had four substitutions (Glu157Gly, Ser193Asp, Ala216Val, and Pro565Ser). These observations suggest that the error-prone PCR had worked well and could be used to generate diversity in the enzyme library.

After the error-prone PCR, a library of mutant chitinase genes was created by DNA shuffling, as described in Materials and methods. Results from the key steps in this procedure are depicted in Fig. 1. The shuffled genes were cloned into pFLAG-CTS, where they are fused to the *E. coli* OmpA signal peptide that directs secretion to the periplasmic space [14]. This greatly facilitates the screening process in the next step.

3.2 Screening for improved chitinases

In the primary screening, chitinase activity was tested by plating transformants on LB agar plates containing 0.2% colloidal chitin (Fig. 2). Approximately 60% of the colonies (293 clones) showed en-

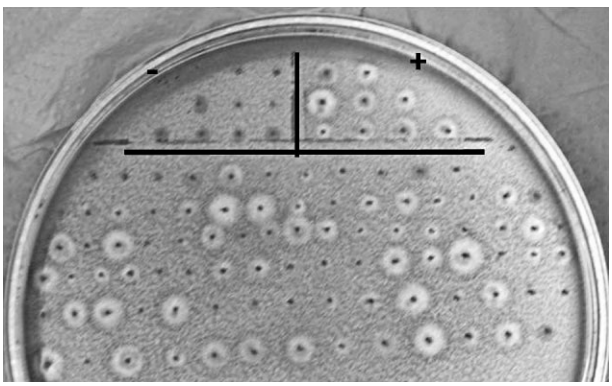

Figure 2. Primary screening on agar plate. *E. coli* transformants were arrayed on an LB/ampicillin plate containing 0.2% colloidal chitin. Colonies producing active chitinases show a clearing zone, after staining with 0.2% Congo Red. Positive (+) and negative (–) controls were plated in the triangles in the upper right and the upper left part of the plate, marked with + and –, respectively. Note that the colonies of the positive control (all expressing the wild-type pFchi8785) show different halo sizes, indicating that there is a limited correlation between halo size and enzyme-specific activity.

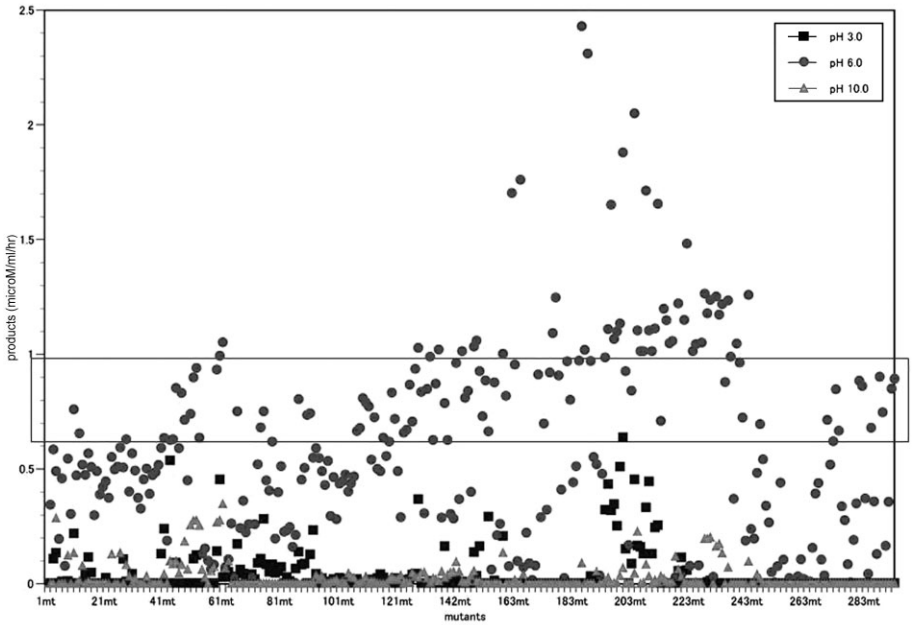

Figure 3. Secondary screening in microtiter plates. Chitinase activity was measured for 293 variants, at three different pH values, using *p*-NP-$(GlcNAc)_2$ as substrate. Activities are expressed as total product release per hour (µM/mL/h). Squares, pH 3; circles, pH 6.0, triangles, pH 10.0. The box indicates the range of activities measured at pH 6.0 for clones expressing the wild-type enzymes (in total 293 samples of each of the two wild-type enzymes were analyzed). Only trace amount of enzyme activity of the wild-type enzymes could be detected at pH 3.0 (0.042 µmol/mL/h), or pH 10.0. (0.030 µmol/mL/h).

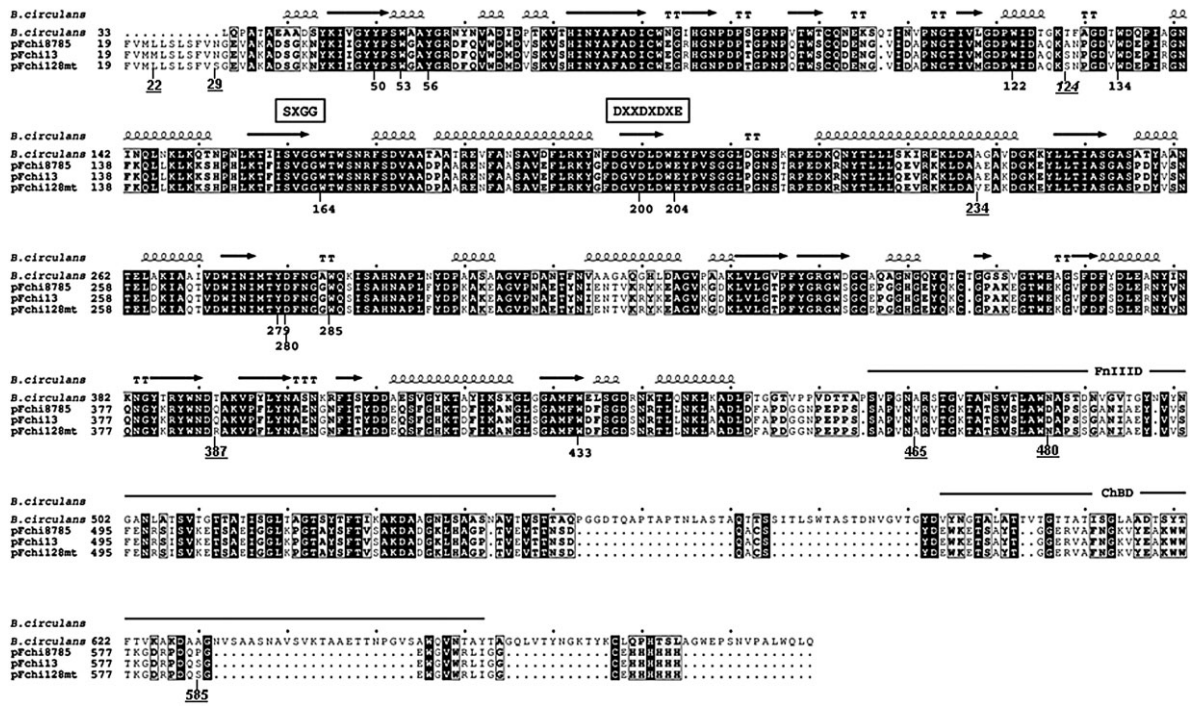

Figure 4. Amino acid sequence alignment of wild-type and mutant *B. licheniformis* chitinases and ChiA1 from *B. circulans*. The picture shows the sequences of recombinant wild-type ChiA from *B. licheniformis* strains DSM13 (pFchi13) and DSM8785 (pFchi8785) and of the selected mutant pFchi128mt. The sequence of the catalytic domain is annotated for secondary structure using the 3-D structure of the catalytic domain of ChiA1 from *B. circulans* as a template [27]. α helices and β strands are displayed as squiggles and arrows, respectively. TT and TTT indicate turns. Fully conserved residues are printed as white characters on black background. Similar but not fully conserved residues are boxed. The amino acid sequences of the three *B. licheniformis* chitinases start with the remaining part of the (predicted) leader peptides (see [14] for further details). Residues that have been reported to be important for catalytic activity of this type of enzymes are numbered without underline [26, 28–31], according to the numbering of the *B. licheniformis* enzymes. Two diagnostic sequence motifs are shown in boxes, above the sequence. The mutated residues in 128mt are marked by underlined sequence numbers. Residues that are different between the two wild-type enzymes are marked by underlined sequence numbers in italics. ChBD and FnIIID indicate the chitin binding domain and the fibronectin type III domain, respectively, as predicted by Pfam, [32]). The sequence alignment was made using CLUSTAL W (1.83) multiple sequence alignment software [33], then ESPript [34] was linked to display the secondary structure of the template selected (*B. circulans* ChiA1; pdb code; 1ITX).

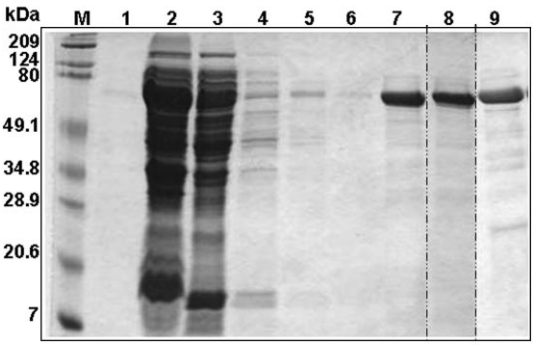

Figure 5. Expression and purification of recombinant chitinases. The picture shows SDS-PAGE gels stained by Coomassie. All lanes concern purification of pFchi13, unless stated otherwise. M, size markers; 1, culture supernatant; 2, cell lysate; 3, flowthrough; 4–6, 40 mM imidazole wash; 7, eluted pFchi13; 8, eluted pFchi8785l; 9, eluted pFchi128mt.

zymatic activity as indicated by the appearance of a clearing zone. Repetitive plating of the positive controls showed considerable variation in halo sizes (Fig. 2), indicating that the halo radius only gave limited quantitative information about activity (enzymes purified from cultures inoculated with wild-type colonies giving large or small clearing zones had identical specific activities; data not shown).

After the primary screening, 293 positive clones were picked from agar plates and subjected to secondary screening in microtiter plates at three different pHs (3.0, 6.0, and 10.0). The results from the secondary screening are shown in Fig. 3. Since obtaining an enzyme with activity at low pH was most interesting for our application, we picked five clones from the three plates that showed relatively high activity at pH 3.0 (43mt, 128mt, 197mt, 199mt, 204mt). These clones were cultured in 10 mL LB supplemented with ampicillin to confirm enzyme activity. Enzyme preparations, obtained from cell lysates, prepared as described in Materials and methods, were then used to study specific activity towards colloidal chitin at pH 3.0. Whereas four of the enzymes showed wild-type activity, one (128mt) could hydrolyze colloidal chitin better than the wild-type at pH 3.0 (approximately three times faster), while showing near wild-type activity at pH 6.0. The 128mt clone contained five residues that differed from both of the wild types: Ser29 (Asn), Val234 (Ala), Arg387 (Gln), Ala465 (Val) and Asn480 (Asp). Two of these mutations (A234V and Q387R) are in the catalytic domain, two are located in the fibronectin type III domain (FnIII) (V465A and D480N) and one (N29S) is located in the remaining part of the N-terminal signal peptide of the chitinase (see legend to Fig. 4 and below for further discussion).

3.3 Analysis of an improved enzyme

To further characterize the potentially improved enzyme, genes encoding the two wild-type ChiA from *B. licheniformis* strains DSM13 and DSM8785, and the gene encoding the 128mt mutant were subcloned into a pFLAG-CTS expression vector such that a hexa-histidine tag was fused to the C terminus of the enzyme. These recombinant chitinases were designated pFchi8785, pFchi13, and pFchi128mt. All recombinant enzymes could be over expressed and affinity purified using Ni-NTA column chromatography to apparent homogeneity as shown in Fig. 5. Both wild-type and mutant chitinases showed optimal activity in the temperature range of 45–60°C, and had an optimal pH for activity at 6 (data not shown), indicating that the mutant generally had properties similar to those of the wild-types. To elucidate in more detail the properties of each enzyme, Michaelis-Menten type kinetic parameters were determined using *p*-NP-chitobiose as a substrate (Table 1). Kinetic parameters were determined from a subset of the data spanning the 0.008–0.18 mM substrate concentration range because substrate inhibition became apparent at higher substrate concentrations (as is quite

Table 1. Kinetic parameters of wild-type chitinases and the selected mutant, determined with *p*-nitrophenyl-chitobiose as a substrate[a)]

Enzyme	K_m (mM)	V_{max} (mM/min)	k_{cat} s^{-1}	k_{cat}/K_m s^{-1}/mM
pH 3.0				
pFChi13	0.10±0.004	0.15±0.004	0.17±0.004	1.69±0.024
pFChi8785	0.11±0.025	0.12±0.033	0.14±0.037	1.36±0.667
128mt	0.06±0.001	0.21±0.059	0.23±0.067	4.10±1.240
pH 6.0				
pFChi13	0.06±0.011	0.38±0.113	0.43±0.126	7.55±0.745
pFChi8785	0.03±0.003	0.28±0.063	0.31±0.070	10.30±3.236
128mt	0.03±0.001	0.59±0.172	0.65±0.191	19.40±4.578

[a)] Assays were carried out as described in material and methods. The SDs were determined from four independent experiments.

common for this type of chitinases [20, 21]). Kinetic analysis revealed that the 128mt mutant was more effective in hydrolyzing *p*-NP-chitobiose than the wild-type enzymes at both at pH 6.0 and 3.0. The overall catalytic efficiency, k_{cat}/K_m of 128mt was approximately 2.7- and 2.3-fold higher than the average value for the wild-type enzymes at pH 3.0 and 6.0, respectively. Table 1 also shows that the two wild-type enzymes have similar properties. The difference between 128mt and each of the wild types is larger than the difference between the wild types.

4 Discussion

An efficient method for directed evolution of chitinases has been established by taking advantage of the ability to secrete *Bacillus* hydrolytic enzymes into the culture medium [14]. Secretion of the chitinases allowed the use of a simple, cheap agar-plate-based first screening step for pre-selection of active enzyme variants only. This pre-screening reduces the number of more laborious microtiter-plate-based enzyme assays required subseqently. Access to such a pre-screen also facilitates the use of higher mutation frequencies, since inactive mutants are discarded before expensive enzyme assays are run. It is important to note that we found that the halo size did not correlate well with enzyme activity, whereas the size of the clearing zone has been used successfully for estimating enzyme activity in other publications [22, 23]. The different sizes of the clearing zones could simply be the result of differences in the expression and/or secretion levels that are caused by local variation on the (hand-picked) plates or the size of the inocula. Thus, the simple plate assay described here was only used to discard clones that did not show any activity at all. In the second screening we use a microtiter-plate-based assay that allowed us to screen for enzyme activity at three different pH values. Clearly, this screening procedure not only identifies improved variants, but is also affected by mutational effects on and variation in protein expression and/or secretion. Therefore, clones that seemed most promising in the microtiter-plate-based screen were cultured on a larger scale and a more optimal temperature with respect to expression for further analysis. This led to selection of one clone producing an improved chitinase as proven by subsequent kinetic characterization. The measurement of enzyme activity in microtiter plates has a number of advantage, mainly because many enzyme properties can be screened for at the same time, as demonstrated in this report, where catalytic activity at both high and low pH could be screened for simultaneously. However, a limitation of the microtiter plate assay is that the plate is not resistant to high temperature or certain organics solvent, unless special plates are used [24].

Since the two starting genes were highly similar (Fig. 4), and since the two wild-type enzymes have similar properties (Table 1), there was no *a priori* reason to include DNA shuffling into the protocol for diversity generation. We included this step to demonstrate that it could be implemented in the new method for directed evolution of chitinases that we present. Interestingly, the selected mutant, 128mt, is in fact most likely the result of shuffling of the two wild-type genes (see Fig. 4; residue 22 in 128mt comes from DSM8785, whereas residues 124 and 585 come from DSM13) as well as from the changes introduced through error-prone PCR.

The chitinases used in this study belong to glycosyl hydrolase family 18 [25] for which there is ample structure-function information, at least for the catalytic domain. Thus, it is easy to get an impression of the approximate structural location of the selected mutations by simply aligning the sequence of the *B. licheniformis* enzymes with a structure-based alignment of family 18 chitinases (as in for example Horn *et al*. [6]) or by aligning the *B. licheniformis* enzymes with the closely related chitinase ChiA1 from *B. circulans* for which the structure is known (Fig. 4). The 128mt mutant contains two mutations in the catalytic domain (A→V at position 234 and Q→R at position 387). Inspection of the ChiA1 structure showed that the A234V mutation affects the C-terminal end of helix four in the $(\beta/\alpha)_8$ barrel of the catalytic domain and is thus located on the side of the barrel that is opposite from the active site. Inspection of the ChiA1 structure further suggested that residue 234 may be involved in the interaction between helix four and helix three of the barrel and this interaction may be affected by the mutation. The other mutation, at position 387, is located in the so-called α+β domain that is inserted in the $(\beta/\alpha)_8$ barrel between strand seven and helix seven [26]. Residue 387 is likely to be positioned on the surface of the protein and point into solution on a side of the α+β domain that is not in contact with the substrate. Inspection of the ChiA1 structure suggested that a change from Gln (or Thr as in ChiA1) to Arg at this surface-located site far from the catalytic center would have no effects other than the charge difference. All in all, there are no obvious explanations for the observed mutational effects, as is often the case for mutants coming out of directed evolution experiments [23, 25, 33]. Probably, the changes in activity are caused by effects that are difficult to capture by

structural inspection, such as subtle long-range structural changes and changes in dynamics (conceivable for the Ala 234→Val mutation) or changes in long-range electrostatic effects (conceivable for the Gln 387→Arg mutation).

The 128mt variant also had two mutations (V465A and D480N) in the FNIII domain, which, remarkably, are the same amino acid substitutions found in ChiA1. *A priori*, mutations in the FNIII domain are not expected to affect activity towards the small oligomeric substrate pNP-chitobiose, but they may affect activity towards certain forms of chitin [31, 32]. Further studies are necessary to study whether this is the case. FNIII domains tend to interact strongly with the catalytic domains as shown by the crystal structure of chitinase A from *Serratia marcescens*, which has an FNIII domain coupled to the N terminus of the catalytic domain [26]. So, at least in the case of chitinase A, it is possible that mutations on the FNIII domain have subtle effects on the catalytic center itself and, thus, perhaps on activity towards pNP-chitobiose. Unfortunately, there is no such structural information for a family 18 chitinase with a C-terminally located FNIII that could shed light on the situation for the *B. licheniformis* enzymes.

Generally, rationalizing the outcome of directed evolution experiments is often difficult. Selected mutants with beneficial properties often contain several mutations with no obvious structural and functional implications. Clearly, the selection for non-obvious but useful mutations is one of the powers of directed evolution. The latter is also illustrated by the present study, where a simple and straightforward procedure for expression, mutation and selection of chitinases resulted in identification of a mutant with improved properties after screening less than 600 colonies only. The method described in this study can be used for directed evolution of chitinases and other hydrolytic enzymes in the future.

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5 References

[1] Coutinho, P. M., Henrissat, B. (Eds.), *Carbohydrate-Active Enzymes: An Integrated Database Approach*. The Royal Society of Chemistry, Cambridge 1999.

[2] Howard, M. B., Ekborg, N. A., Weiner, R. M., Hutcheson, S. W., Detection and characterization of chitinases and other chitin-modifying enzymes. *J. Ind. Microbiol. Biotechnol.* 2003, *30*, 627–635.

[3] Koga, D., Mitsutomi, M., Kono, M., Matsumiya, M., Biochemistry of chitinases. *EXS* 1999, *87*, 111–123.

[4] Bhattacharya, D., Nagpure, A., Gupta, R. K., Bacterial chitinases: Properties and potential. *Crit. Rev. Biotechnol.* 2007, *27*, 21–28.

[5] Dahiya, N., Tewari, R., Hoondal, G. S., Biotechnological aspects of chitinolytic enzymes: A review. *Appl. Microbiol. Biotechnol.* 2006, *71*, 773–782.

[6] Horn, S. J., Sikorski, P., Cederkvist, J. B., Vaaje-Kolstad, G. *et al.*, Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. *Proc. Natl. Acad. Sci. USA* 2006, *103*, 18089–18094.

[7] Hayes, M., Carney, B., Slater, J., Bruck, W., Mining marine shellfish wastes for bioactive molecules: chitin and chitosan. Part B. Applications. *Biotechnol. J.* 2008, *3*, 878–889.

[8] Williams, G. J., Nelson, A. S., Berry, A., Directed evolution of enzymes for biocatalysis and the life sciences. *Cell. Mol. Life Sci.* 2004, *61*, 3034–3046.

[9] Farinas, E. T., Bulter, T., Arnold, F. H., Directed enzyme evolution. *Curr. Opin. Biotechnol.* 2001, *12*, 545–551.

[10] Stemmer, W. P., DNA shuffling by random fragmentation and reassembly: *In vitro* recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA* 1994, *91*, 10747–10751.

[11] Kurtzman, A. L., Govindarajan, S., Vahle, K., Jones, J.T. *et al.*, Advances in directed protein evolution by recursive genetic recombination: Applications to therapeutic proteins. *Curr. Opin. Biotechnol.* 2001, *12*, 361–370.

[12] Patten, P. A., Howard, R. J., Stemmer, W. P., Applications of DNA shuffling to pharmaceuticals and vaccines. *Curr. Opin. Biotechnol.* 1997, *8*, 724–733.

[13] Arnold, F. H., Volkov, A. A., Directed evolution of biocatalysts. *Curr. Opin. Chem. Biol.* 1999, *3*, 54–59.

[14] Yamabhai, M., Emrat, S., Sukasem, S., Pesatcha, P. *et al.*, Secretion of recombinant *Bacillus* hydrolytic enzymes using *Escherichia coli* expression systems. *J. Biotechnol.* 2008, *133*, 50–57.

[15] Veith, B., Herzberg, C., Steckel, S., Feesche, J. *et al.*, The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *J. Mol. Microbiol. Biotechnol.* 2004, *7*, 204–211.

[16] Cirino, P. C., Mayer, K. M., Umeno, D., Generating mutant libraries using error-prone PCR, in: Arnold, F. H., Gerogiou, G. (Eds.), *Directed Evolution Library Creation,* vol. 231. Humana Press, Totowa 2003, pp. 3–9.

[17] Joern, J. M., DNA shuffling, in: Arnold, F. H., Gerogiou, G. (Eds.), *Directed Evolution Library Creation,* vol. 231. Humana Press; Totowa 2003, pp. 85–89.

[18] Sambrook, J., Russell, D. W., Molecular Cloning a Laboratory Manual, 3 edn, Cold Spring Harbor, New York 2001.

[19] Zhao, H., Arnold, F. H., Optimization of DNA shuffling for high fidelity recombination. *Nucleic Acids Res.* 1997, *25*, 1307–1308.

[20] Brurberg, M. B., Nes, I. F., Eijsink, V. G., Comparative studies of chitinases A and B from *Serratia marcescens*. *Microbiology* 1996, *142*, 1581–1589.

[21] Synstad, B., Gaseidnes, S., Van Aalten, D. M., Vriend, G. *et al.*, Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase. *Eur. J. Biochem.* 2004, *271*, 253–262.

[22] Fan, Y., Fang, W., Xiao, Y., Yang, X. *et al.*, Directed evolution for increased chitinase activity. *Appl. Microbiol. Biotechnol.* 2007, *76*, 135–139.
[23] Lee, S. H., Ryu, E. J., Kang, M. J., Wang, E.-S. *et al.*, A new approach to directed gene evolution by recombined extension on truncated templates (RETT). *J. Mol. Catal. B. Enzym.* 2003, *26*, 119–129.
[24] Arnold, F. H., Georgiou, G. (Eds.), *Directed Enzyme Evolution: Screening and Selection Methods*. Humana Press, Totowa 2003.
[25] Henrissat, B., Classification of chitinases modules. *EXS* 1999, *87*, 137–156.
[26] Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B. *et al.*, Crystal structure of a bacterial chitinase at 2.3 Å resolution. *Structure* 1994, *2*, 1169–1180.
[27] Matsumoto, T., Nonaka, T., Hashimoto, M., Watanabe, T., Mitsui, Y., Three-dimensional structure of the catalytic domain of chitinase A1 from *Bacillus circulans* WL-12 at a very high resolution. *Proc. Jpn. Acad. Ser. B* 1999, *75*, 269–274.
[28] van Aalten, D. M., Synstad, B., Brurberg, M. B., Hough, E. *et al.*, Structure of a two-domain chitotriosidase from *Serratia marcescens* at 1.9-Å resolution. *Proc. Natl. Acad. Sci. USA* 2000, *97*, 5842–5847.
[29] Watanabe, T., Ariga, Y., Sato, U., Toratani, T. *et al.*, Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. *Biochem. J.* 2003, *376*, 237–244.
[30] Watanabe, T., Kobori, K., Miyashita, K., Fujii, T. *et al.*, Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* 1993, *268*, 18567–18572.
[31] Watanabe, T., Uchida, M., Kobori, K., Tanaka, H., Site-directed mutagenesis of the Asp-197 and Asp-202 residues in chitinase A1 of *Bacillus circulans* WL-12. *Biosci. Biotechnol. Biochem.* 1994, *58*, 2283–2285.
[32] Finn, R. D., Tate, J., Mistry, J., Coggill, P. C. *et al.*, The Pfam protein families database. *Nucleic Acids Res.* 2008, *36* (Database issue), D281–288.
[33] Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R. *et al.*, Clustal W and Clustal X version 2.0. *Bioinformatics* 2007, *23*, 2947–2948.
[34] Gouet, P., Robert, X., Courcelle, E., ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. *Nucleic Acids Res.* 2003, *31*, 3320–3323.

