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Phage display selects for amylases with improved low pH starch-binding

Raymond M.D. Verhaert [a,1], Jules Beekwilder [b,2], René Olsthoorn [b], Jan van Duin [b], Wim J. Quax [a,*]

[a] *Pharmaceutical Biology, University Centre for Pharmacy, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands*
[b] *Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

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Abstract

Directed evolution of secreted industrial enzymes is hampered by the lack of powerful selection techniques. We have explored surface display to select for enzyme variants with improved binding performance on complex polymeric substrates. By a combination of saturation mutagenesis and phage display we selected α-amylase variants, which have the ability to bind starch substrate at industrially preferred low pH conditions. First we displayed active α-amylase on the surface of phage fd. Secondly we developed a selection system that is based on the ability of α-amylase displaying phages to bind to cross-linked starch. This system was used to probe the involvement of specific β-strands in substrate interaction. Finally, a saturated library of α-amylase mutants with one or more amino acid residues changed in their Cβ4 starch-binding domain was subjected to phage display selection. Mutant molecules with good starch-binding and hydrolytic capacity could be isolated from the phage library by repeated binding and elution of phage particles at lowered pH value. Apart from the wild type α-amylase a specific subset of variants, with only changes in three out of the seven possible positions, was selected. All selected variants could hydrolyse starch and heptamaltose at low pH. Interestingly, variants were found with a starch hydrolysis ratio at pH 4.5/7.5 that is improved relative to the wild type α-amylase. These data demonstrate that useful α-amylase mutants can be selected via surface display on the basis of their binding properties to starch at lowered pH values. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Enzymes; Polymeric substrates; α-Aamylase; Hydrolysis

* Corresponding author. Tel.: +31-50-3632558; fax: +31-50-3633000.
E-mail address: w.j.quax@farm.rug.nl (W.J. Quax).
[1] Present address: Cargill R&D Europe Lelyweg 31, 4612, PS Bergen op Zoom, The Netherlands.
[2] Present address: Department of Molecular Biology, Plant Research International, P.O. Box 16, 6700, AA Wageningen, The Netherlands.

1. Introduction

In recent years the possibilities for creating genetic diversity have been expanded enormously with the advent of the PCR technique (error prone PCR, PCR with spiked oligo's, staggered extension process) and DNA shuffling (Crameri et al., 1997; Zhao et al., 1998; Matsumura et al.,

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1999). The combination of gene pool diversification and selection for function is nowadays considered as one of the strongest strategies for protein engineering that is collectively termed 'directed evolution'. In evolution two processes play a key role: diversification of the gene pool and selection of the best individual of that pool. Most of the newly described PCR techniques have been exemplified on intracellular enzymes with in vivo selectable functions, such as β-lactamase that can be selected for by increasing the antibiotic concentrations (Stemmer, 1994). For non-essential or for extracellular proteins, which represent the majority of industrial enzymes, an in vivo functional selection is less obvious. The industrially important enzyme α-amylase is secreted from the cell and no cell bound enzymatic activity can be detected in the host *Bacillus licheniformis*. Living cells do not take up complex insoluble substrates, such as starch, cellulose, xylanase, and lipids and therefore enzymatic conversion without exception occurs in the extracellular medium. This leads to a loss of the physical link between the genotype—the DNA—and the phenotype—the enzymatic activity—that impairs the possibility for a selection.

In the field of antibody engineering the phage display technique has been well developed as a selection technique that maintains the coupling between externally displayed peptides and the genotype (for review see Hoogenboom et al., 1998). Phage display has also been used successfully to select for complete proteins, such as better ligands of extracellular receptors (Lowman and Wells, 1993), however in the field of enzyme engineering the method of phage display has been barely applied (Jestin et al., 1999; Demartis et al., 1999). A reason for this limitation may be that phage display typically selects for a binding interaction between the protein and its target, while for the improvement of an enzyme apart from binding also catalysis is needed. It follows that phage display selection may be successfully used for those enzymes where the rate limitation is in substrate binding (K_m) rather than in maximal velocity (V_{max}). In many industrial processes involving the conversion of complex polymeric substrates indeed the binding of an enzyme to the substrate is a particularly rate limiting step. Often these enzymes have evolved specific substrate binding domains at a location remote from the active site of the enzyme.

In order to explore this opportunity, we decided to investigate the possibility to use phage display for a distinct problem in the grain wet milling industry. α-Amylase (1,4 α-D-glucanhydrolase, EC 3.2.11) catalyses the hydrolysis of α-1,4 glucan linkages in starch to produce larger oligosaccharides and maltose. The enzyme is produced by a variety of microorganisms including *Bacillus* and *Aspergillus*. The enzyme from *B. licheniformis* is very heat stable and therefore it is particularly suited for the large-scale liquefaction of cornstarch in industry. In the liquefaction process, the α-amylase is mixed with starch slurry and heated to a temperature of 110 °C in order to obtain liquefaction. Before this process the pH of the slurry has to be adjusted from pH 4.5, the natural value, to a value between pH 5.8 and 6.5 (Crabb and Mitchinson, 1997). The process cannot be operated properly at a value below a pH of 5.9, since the α-amylase does not form oligosaccharides below that value. After the liquefaction the pH of the starch has to be adjusted again—now to pH 4.2—in order to provide the conditions for the saccharification process by the fungal exo 1-4 glycanohydrolase, that splits the oligosaccharides into glucose units. Therefore the availability of an α-amylase, which has a good performance at low pH, would eliminate process steps and reduce the costs for chemicals and ionic exchange resins related to these pH adjustments.

The structure of *B. licheniformis* α-amylase has been resolved and the co-ordinates have been released recently (Machius et al., 1995; Hwang et al., 1997). The αβ-barrel central domain including the active site residues has been extensively discussed. The C-terminal region is characterized by a number of β-sheet regions designated Cβ1 to Cβ8; however, to this domain of the enzyme no function has been ascribed (Machius et al., 1995). The α-amylase shows homology with other α-amylases and with the well-studied cyclodextrin transferases. Notably in the latter enzymes the C-terminal domain is significantly extended and in this domain β-sheet residues have been identified

that are responsible for the interaction with starch (Penninga et al., 1996). Despite the fact that no specific starch-binding domain has been assigned to the *B. licheniformis* α-amylase structure, functional studies show that the enzyme can scroll along the starch surface to break down the complete polymer into oligosaccharides (Helbert et al., 1996). This suggests a specific interaction between the enzyme and its complex starch substrate distinct from the hydrolytic reaction. In most polysaccharide degrading enzymes the catalytic and substrate binding domains are separated, implying that the starch-binding property of the protein can be independent from the catalytic action of the enzyme. Therefore we decided to study if the C-terminal domain of the *B. licheniformis* α-amylase is indeed involved in starch-binding and to explore the possibility to develop a phage display selection method for α-amylase mutants which have altered starch-binding. The starch-binding property was, without relating this property to a specific part of the protein molecule, already used to develop a large-scale purification procedure, which involves affinity chromatography to raw starch or cross-linked starch (Weber et al., 1976; Rozie et al., 1991; Satoh et al., 1993; Somers et al., 1995). Interestingly at pH 4.5 the enzyme shows a reasonable hydrolytic activity on the small substrate heptamaltose, whereas the activity on polymeric starch is zero. Based on this observation we hypothesized that a decreased starch-binding capacity hampers the application of α-amylase at pH values lower than six. In the past the complete gene was subjected to low intensity random mutagenesis and the variants with altered halo formation capacity were selected. The mutations selected in those experiments reside in the αβ-barrel central domain (Quax et al., 1994; Mitchinson et al., 1998). We decided to address specifically the C-terminal domain by a saturation mutagenesis method that probes every residue over a limited region. In order to explore a large sequence space a powerful selection is essential. The phage display technique offers such a possibility.

The technique was originally developed by Smith (1985), who showed that small peptides can be expressed on the surface of the filamentous phage fd as a fusion to the N-terminus of g3p coat protein resulting in the 'display' of that peptide. It was shown that it is possible to select for a phage encoding a specific peptide by affinity binding. It has been reported that a single phage particle can be selected out of a population of 10^9 by multiple rounds of selection and enrichment (For a review see Cortese et al., 1994). The selection of a desired peptide, e.g. by binding it to an antibody, automatically leads to the co-selection and isolation of the DNA that codes for the peptide. Initially the application of this technique was restricted to small peptides. Later it was found that protein domains (Roberts et al., 1992) and even complete proteins could be functionally expressed as fusion proteins to g3p. Also β-lactamase was displayed on the surface of phage fd as a fusion with g3p coat protein. A suicide inhibitor was used to selectively enrich a mutant enzyme (Soumillion et al., 1994). Recently we have demonstrated that also penicillin G acylase, an enzyme requiring post-translational processing in the periplasm, can be expressed actively on the surface of a phage particle (Verhaert et al., 1999).

So by displaying α-amylase on the surface of a phage and by setting up a selection system that discriminates between α-amylases (linked to phages) on their starch-binding ability, we here demonstrate that it is possible to isolate amylases with improved substrate binding. Obviously this method is not restricted to α-amylase. We foresee that in a similar fashion improved variants of polymeric substrate converting enzymes such as cellulases, xylanases, chitinases, ligninases, lipases and proteases can be selected.

2. Experimental

2.1. Materials

The following bacterial strains were used: *E. coli* GM-1 (Miller et al., 1977), *E. coli* TG1: Sup E, K 12 Δ(lac-pro), thi, hsd D5/F', traD 36, pro AB, lacIq, lac Z ΔM15 and *E. coli* HB 2151: K12 Δ(lac-pro), ara, nal^r,thi/F', pro AB, lacIq, lac Z ΔM15. Bacteriophage M13KO7 and phagemid pCANTAB 5E were bought from Pharmacia,

phagemid pMc/NdeI was used as described (Stanssens et al., 1989). Purified α-amylase from *B. licheniformis* was kindly provided by Gist-brocades N.V. (Delft, The Netherlands). Raw cornstarch granules were obtained from AJB, Zaandam, The Netherlands (Trade name: Maizena).

All primers were synthesized by an in-house facility of the Leiden University. The nucleotide sequence coding for a restriction site is given in lowercase typing.

2.2. Methods

2.2.1. Design and construction of plasmids for α-amylase display and selection

The *B. licheniformis* α-amylase gene from plasmid pLAT3 (Sanders et al., 1985) was amplified using oligonucleotides Af1 (5′-C TTC TTG CTG CCT CAg gcc cag ccg gcc ATG GCA AAT CTT AAT GGG-3′) and Ar1 (5′-CC GTC CTC TCT Ggc ggc cgc TCT TTG AAC ATA AAT TG-3′). The amplified DNA fragment was digested with *Sfi*I and *Not*I, and cloned into vector pCANTAB 5E. This resulted in plasmid pRα1. The lacZ inducible sequence now codes for an α-amylase fused to the g3p coat protein of bacteriophage fd. Using *Hind*III/*Not*I digestion the α-amylase coding sequence was inserted in expression/display vectors (i) that have a his tag interlaced between the α-amylase and the g3p (ii) that result in display on g8p or (iii) that have the amber stop codon between the α-amylase and the phage coat protein removed (Fig. 1, see Verhaert et al., 1999).

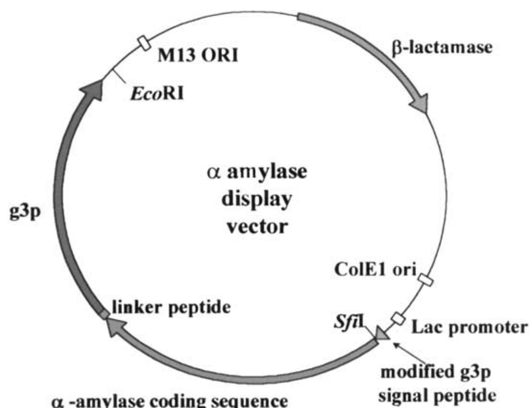

Fig. 1. Plasmids used for display and selection of α-amylase. The plasmid was derived from pCANTAB 5E and pPGA-H-g3p (24). It is a phagemid vector with both ColE1 and M13 origin of replication. The gp3 signal sequence was modified as described in (24).

2.2.2. Phage rescue, titer determination, phage electrophoresis and Western blotting

Phage particles were rescued similar to the procedure described earlier (Verhaert et al., 1999). After infection with M13KO7 phage, cells were spun down at $2500 \times g$ for 5 min, and resuspended in 100 ml 2xYT medium with ampicillin and kanamycin. Cultures were shaken (250 rpm) overnight at 28 °C. From the supernatant of the overnight culture, phagemids were precipitated with 4% PEG6000/0.5 M NaCl. Pellets were resuspended in 1 ml 10 mM Hepes pH 7.

To determine the titer routinely, phagemid solutions were diluted in 1 ml *E. coli* GM-1 (Miller et al., 1977) cell suspension (A650 = 0.4). The very low titer values obtained after selection were determined by the dilution of 25–50 μl of eluens with ten volumes of cells. This procedure was developed because a high concentration of adsorption buffer inhibits infection. The mixture was left at room temperature for 15 min, and plated on antibiotic-containing plates.

Phagemid samples were separated by overnight electrophoresis in 2% agarose, buffered by 25 mM Tris/250 mM glycine, pH 8.6 at 20 V as described earlier for penicillin G acylase displaying phage particles (Verhaert et al., 1999). Part of the gel was briefly soaked in a 0.5% SDS solution, and blotted onto nitrocellulose (Schleicher and Schuell). α-Amylase on the blot was detected by a polyclonal antiserum against α-amylase from *B. licheniformis* raised in rabbits and alkaline phosphatase-linked *anti*-rabbit goat antibodies (Sigma). A comparable part of the gel, loaded with the same samples was stained by ethidium bromide in a 0.1 N NaOH solution and destained in water, to detect DNA.

Phages were filter dialyzed with Microsep 300K (Filtron) centrifugal concentrators. In a control experiment they proved to be fully permeable for α-amylase. Phage solution (10^{12} phages) was loaded to the concentrator. The concentrator was

spun at $5000 \times g$. After the run, the filtrate was removed and analyzed for phages and enzyme activity.

2.2.3. Identification of amino acid residues for randomization, construction of the phage library and analysis for its diversity

The residues for randomization were selected by visual inspection of the 3D structure of the *B. licheniformis* enzyme for β-sheet quality as present in the Brookhaven Protein Data bank (1bpl). The sequences selected were also analyzed for homology with the amino acid sequence of other α-amylases and glucosidases.

A library was constructed on the fourth β-sheet, Cβ4, as follows: A spiked oligo (Bio744: 5′-CATTAATAACAGACGGACCCGGTGGG-GCAAAGCGAATGTATGTCGGCCGGCAA-AAC-3′) was prepared by 25% randomization of each nucleotide, which corresponds to the coding sequence for residues 433 to 439 (underlined sequence). Then two fragments were generated by overlapping PCR (Expand™ high fidelity system Boehringer). A 5′ fragment was synthesized using a set of two primers: Bio619 (5′-GG CAA TCG CTT GAg tcg acT GTC-3′, containing a *Hinc*II site) and Bio743 (5′-GTCCGTCTGTTATTAAT-GCC-3′) on plasmid pRA5amb3. A 3′ fragment was synthesized using a second set of primers, Bio744 and Bio561 (5′-C GAA Tag atc tTC ATT AAA GCC AG-3′) on plasmid pRAamb3. Both fragments were isolated from gel over Qiagen columns and approximately 10E10 molecules of each fragment were combined in a second reaction by overlapping PCR (Expand™ high fidelity system, Boehringer) using primers Bio561 and Bio619. The resulting library was reamplified up to 25 μg, digested with SalI and PstI and inserted into the *Sal*I/*Pst*I sites of plasmid pRα-His-Amb-g3p. The phagemid library was generated by transformation of PEG/DMSO processed cells (Verhaert et al., 1999). Phages were rescued as described above using 250 μl library cells as the inoculum, 100 ml LB with 2% glucose and 0.1 μg/ml ampicillin as growth medium until the OD became 0.4 and M13KO7 (2×10^{11} colony forming units) were added. After incubation (1 h) cells were collected and resuspended in 100 ml 2xYT medium containing kanamycin and ampicillin and grown overnight (28 °C). Cells were removed and phagemids precipitated with PEG/NaCl. Phage particles were resuspended in 5 ml 10 mM Tris–HCl pH 7.0/1 mM EDTA and filtered through a 0.45 μm filter to remove residual cells.

The diversity of the library was checked by dideoxy sequencing of randomly picked individual colonies. From a set of 32 mutants 19% had one amino acid change, 31% had two changes, 22% had three, 13% had four, 16% five, $<3\%$ six, $<3\%$ seven. These values correlate well with the theoretical percentages of 15, 28, 28, 17, 4, 0.5, 0.1, respectively. Wild-type nucleotide sequences were present in 10% of the cases, while this number should be $\sim 3.5\%$ based on the design of the spiked oligonucleotide. This effect may result from incomplete digestion and religation of the recipient vector pRα-His-Amb-g3p.

The integrity of the different constructs was also screened using the possibility of α-amylase producing cells to form halos on either LBZ (LB medium with 1.5% bactoagar and 1% starch) or MMZ plates (M9 salts: 12.8 g l^{-1} $Na_2HPO_4.7H_2O$, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1.0 g l^{-1} NH_4Cl; 50 μg l^{-1} thiamine, 50 mg l^{-1} $MgSO_4$, 1.5% bactoagar, 1% starch).

2.2.4. Selection procedures: affinity chromatography and quick select

Starch (25 mg) was suspended in 0.5 ml 100 mM sodium acetate (NaAc) buffer (pH 4.5–6.0). The starch was spun down (1 min at $14000 \times g$) and the supernatant removed. Then the material was preincubated with 0.5 ml adsorption buffer (100 mM NaAc pH 4.5–6.0, 1% ovalbumin, 0.2% Tween 20, 20% glycerol) on ice for 15–20 min. Subsequently, either enzyme or phagemids were added in a 0.1 ml volume and left on ice for 20 min. Non-adsorbed enzyme or phages were removed by brief centrifugation (1 min at $14000 \times g$), after which the starch material was washed three times with ice-cold adsorption buffer. Finally, bound enzyme or phages were eluted by incubation at 70 °C for 5 min in 0.5 ml adsorption buffer at pH 5–6.0. Starch material was removed by centrifugation. Eluens was collected

and a sample was taken for titer and composition analysis.

For an additional round of selection the phage suspension was not amplified but used directly on a new, freshly prepared affinity material. The suspension was adjusted to the desired pH by the addition of 5–25 μl 3 M NaAc pH 4.7.

2.2.5. Analysis of mutants: halo formation and preparation of the periplasmic proteins

On plate the presence of an intact α-amylase copy was determined using starch (Sigma) in either LBZ or MMZ plates. Plasmid DNA from isolated mutants selected in *E. coli* suppressor cells (TG1) was transformed to HB2151 cells and selected for growth on ampicillin and nalidixic acid. A colony was picked and grown overnight. From part of this culture the DNA was isolated and checked by restriction analysis. Two hundred microliters of the same culture was used to inoculate 10 ml of 2xYT, 0.1% glucose, ampicillin medium and grown for 3 h at 28 °C at 300 rpm. Then 5 ml of the same medium with an additional 3 mM IPTG was added and the cells were grown overnight (25 °C, 250 rpm). The cells were pelleted and incubating the cells on ice for 10 min in 500 μl PBS containing 1 mM EDTA liberated periplasmic proteins. Cells were removed by centrifugation (10 min at $14000 \times g$, 4 °C). The supernatant containing the desired periplasmic proteins was collected and stored frozen. α-Amylase was purified using immobilization on Ni^{2+}-nitrolotriacetate agarose (QIAgen) and the amount was determined using the Bradford method (Verhaert et al., 1999).

2.2.6. Enzyme assays: activity on EPS, modified starch and soluble starch

The activity of the enzyme on the small nitrophenyl releasing substrate EPS (4-nitrophenyl-α-D-maltoheptasoide-4,6-O-ethylidene from Boehringer) was carried out following the protocol supplied by the supplier. This continuous assay determines the α-amylase catalyzed hydrolysis of a maltoheptasoide into oligosaccharides. The presence of α-glucosidase liberates the nitrophenyl moiety. The EPS assay was carried out at pH 4.5 and 7.5.

Hydrolysis of starch was routinely measured by the degradation of modified starch (Phadebas, Pharmacia Sweden). In that assay the hydrolysis of the cross-linked starch liberates a blue dye, allowing the determination of enzyme activity on its natural substrate. This activity was related to reference activities presented in the datasheet from the supplier. The starch assay was carried out at pH 4.5 and 7.5.

The starch hydrolysis reaction was quantified using the Phadebas blue starch method. Six hundred milligrams of Phadebas was suspended in 12 ml water. Typically 5–20 μl of enzyme solution was added to 400 μl of this suspension. The suspension was placed at 43 °C and 60 μl samples were withdrawn at 3.5 min intervals, added to a microtube containing 15 μl of 0.5 M NaOH and vortexed immediately. After collecting four samples from each enzyme incubation, the amount of blue starch solubilized by enzymatic hydrolysis was measured by OD620 determination of the supernatant after the addition of 825 μl water to this microtube and centrifugation. The rate of the reaction was determined by linear regression of OD measurements. Care was taken to measure under initial, substrate saturating, conditions. All determinations were carried out at least in duplicate. Measurement at pH 4.5 was carried out after the addition of 20 μl 2 M sodium acetate buffer of that pH to the 400 μl Phadebas suspension.

3. Results

3.1. Display of α-amylase of B. licheniformis on phage fd

The α-amylase gene of *B. licheniformis* was isolated from the plasmid pLAT3 (27) using the primers Af1 and Ar1 and incorporated in the pCANTAB 5E plasmid yielding a g3p fusion (pRα1, Fig. 1). To ensure proper transport of the g3p-amylase fusion the modified g3p signal sequence with an optimised maturation site that has been used successfully for the display of penicillin acylase was used (Verhaert et al., 1999). *E. coli* non–amber suppressor cells were transformed

with the vector and after growth active α-amylase was detected in the periplasm of these *E. coli* cells.

Subsequently phagemids of the same vector were rescued in *E. coli* suppressor cells. After electrophoresis separated phages were immunostained with rabbit anti-α-amylase-antibodies, or with ethidium bromide to visualise the DNA inside the phage particles. As expected the α-amylase comigrates with the DNA. This strongly suggests that the α-amylase is covalently linked to the phagemid.

This result was further substantiated by the use of a centrifugal concentrator, which can be used to dialyse phages. Phage particles (20 MDa) are retained by the 300 kDa filter but free α-amylase (45 kDa) is expected to pass. A solution of pRα1 phages was loaded into the upper compartment of the concentrator and centrifuged until approximately 50% of the volume had passed the filter. Indeed, the filter retained the phages as well as the enzymatic activity, indicating that the enzyme is linked to the phage.

In order to facilitate handling of the α-amylase the gene was transferred to display vector (pRα-His-Amb-g3p), which has a collagenase-sensitive site (GPGGP) and a $(His)_6$-tag engineered between the α-amylase and the g3p. Immunostaining of the enzyme and the DNA indicated that also these phage particles display the α-amylase on their surface.

The phagemid solution was found to be enzymatically active and enzyme units were determined by a regular α-amylase assay. These values were converted into a turnover rate (V, mol of product formed per mol phagemid per second) by incorporating the molecular weight of phagemids (2.5×10^9 mg mol^{-1}, see Model and Russel, 1988). The turnover number of the phagemids was found to be 8.7 s^{-1}. By comparing the turnover rate of the phagemids with that of the free enzyme (185 s^{-1}), it can be calculated that approximately 5% of the phage particles carry a copy of the enzyme linked via their g3p coat protein. Fusion of α-amylase to g8p and g6p did also result in phage-bound amylase, however, the display frequency was much lower with 0.8 and 0.1%, respectively.

3.2. Affinity selection of α-amylase and phagemids carrying α-amylase

Amylase can be purified by affinity chromatography (Weber et al., 1976). This procedure involves the binding of the enzyme to raw starch or cross-linked starch. Typically the enzyme is bound to the starch at 0 °C and it is eluted by a temperature increase to 70 °C.

In our experimental setup comparable activities of free α-amylase and pRα-His-Amb-g3p phagemid α-amylase were incubated with cross-linked starch at 0 °C for 20 min. After this period 70–80% of the activity was cleared from the supernatant both for free and for phage bound α-amylase. The free enzyme was found to bind very tightly to the starch at 0 °C: 10% of the activity was washed off by a first wash, 6% by a second wash and another 3% by a third wash at 0 °C. Elution of the amylase was performed by heating the starch at 70 °C. This released all bound activity in the case of the free enzyme. Typically 50% of the enzyme activity was recovered following a complete procedure including three rinsing steps. The binding and elution of the phage enzymes to the starch is expected to be less efficient than that of the free enzyme since the α-amylase contains a large proteinaceous load in that case. However, in an experiment following the same adsorption–elution protocol as described above we were able to still recover 6% of the phage-enzyme related activity. Thus both free enzyme as well as phagemid-linked enzyme can be immobilised by this method and eluted by temperature elevation. These data suggest that the binding of the phage-displayed α-amylase is sufficiently strong to allow selection out of a pool of non-binding phages.

This was tested by a competition experiment between α-amylase phages and control phages. The α-amylase phages were derived from the pα-His-g3p phagemids (ampicillin resistant) and the control phages were pMc phagemids (chloramphenicol resistant) (Stanssens et al., 1989). A mixture of both phagemids was incubated with cross-linked starch, washed three times and eluted. This procedure was repeated once. The titres of pα-His-g3p and pMc phagemids were

Table 1A
Two step affinity selection of α-amylase-carrying phagemids vs. non-specific phagemids on starch: phagemid ratio and α-amylase activity

		Phagemid ratio α-amylase: control	Activity (%)
1st Round	Applied	20:1	100
	Non-adsorbed	10:1	50
	Eluted	100:1	25
2nd Round	Applied	100:1	25
	Non-adsorbed	70:1	6
	Eluted	70 000:1	6

determined from samples of each step of the procedure. The selectivity of the starch material (being either cross-linked starch or raw starch) is apparent from the ratio of pα-His-g3p to pMc. This was found to increase five fold (from 20 to 100, see Table 1A).

Thus the selection of pα-His-g3p over pMc is due to the presence of α-amylase on the surface of the pα-His-g3p phagemid since both phagemids consist of identical proteins, except for the α-amylase. These data are also supported by competition experiments between control phages and phages, which had their α-amylase coupled to g8p or g6p.

3.3. Optimisation of the phage enzyme selection procedure

A five-fold enrichment may not suffice for a potent selection procedure. Therefore we further elaborated on the principle of repeated rounds of selection in combination with an intermediate amplification of the selected phage particles. After four selection/amplification rounds the ratio between α-amylase displaying and control phagemids had not changed significantly, suggesting that no further selection took place. Colonies from the fourth round were tested for vector integrity by restriction digestion and DNA sequence analysis. It was found that a deletion mutant had taken over the α-amylase population in the third and fourth amplification rounds and in the second round already 50% of the phagemids contained a deletion. This result indicates that the amplification 'selects' for mutants that have lost their insert by outgrowing those that retain the complete α-amylase-g3p sequence as often observed with plasmids (for review see Balbinder, 1993). This rapid loss of almost the entire α-amylase insert made us decide to omit the growing/amplification rounds in the procedure. Instead, a large phagemid pool (4×10^{12}) was used to start the isolation of a proper binder by successive rounds of binding and elution. Thus the phages that were eluted during the first round of selection were used in a second one without intermediate amplification. This simplified procedure improves the selection indeed, since after a second round of selection the ratio of pα-His-g3p/pMc is increased from 20 (initially) to 70000 (Table 1A). Thus the α-amylase phages have been enriched by a factor of 3500. This observation is confirmed by α-amylase enzyme activity measurements (Table 1B).

The limits of the quick select method were tested in a pilot experiment in which 10^{10} α-amylase displaying phage particles were used at the start of the selection protocol. It was possible to

Table 1B
Stability of phagemid particles at lowered pH

pH	Enrichment factor	Phagemid infectivity (%)	
		After 1 h at 4 °C	After 5 min at 70 °C
6.0	1000–3000	N.D.	N.D.
5.5	N.D.	100	50
5.0	100	75	0.5
4.5	5	60	0.5

lead the phages through up to seven rounds of binding and elution. After those seven rounds approximately 10^3 had survived the complete procedure.

Using other polysaccharide carriers the specificity of the selection on starch was tested. Besides cross-linked starch only starch granules were found to be positive in binding amylase phages. Non-starch solids like cellulose were not able to bind amylase phages confirming that phage binding is starch-specific.

The binding of α-amylase phage particles to raw starch granules can be done at different pH values. However, the enrichment decreased a factor of two hundred when the protocol was carried out at pH 4.5. This decrease is not caused by a loss of viability of the incubated phages at that pH (Table 1B), but by the combination of the increased temperature and the lowered pH during phage elution: less than 1% of the phages survive the 5 min incubation at 70 °C and pH 4.5.

3.4. α-Amylase phagemid library

The C terminal domain of α-amylase may be involved in the binding of the protein to starch. In particular, the fourth β-strand of this domain (Cβ4) may be the location for close contacts between the glucose residues and the protein based on inspection of the 3D structure. We decided to address the residues that are in Cβ4 (433GGAKRMY439) for saturation mutagenesis and selection. PCR with primers that are partially randomised for residues 433–439 was used to create a library of 5×10^4 bacteria.

Based on the design of the primers we expect single amino acid mutants on 15% of the particles, double mutants in Cβ4 on 28% of the phages and wild type α-amylase on 3.4% of the phages. The other phages will have α-amylase with three or more amino acid residues changed. A distribution according to this design was indeed found upon sequencing 32 randomly picked phagemids (Table 2). Each specific single amino acid mutant will be present in a low frequency since in all seven positions, each of the 20 amino acids can be incorporated. The chance of finding e.g. Phe at position 439 is 0.1%. Each double mutant is expected to occur on $\sim 0.003\%$ of the phages. From the data of Table 2 in combination with the size of the library it can be calculated that apart from all single mutations the library also contains all double mutants in this region with at least a 4-fold redundancy.

3.5. Specific mutations in the starch-binding domain are selected by phage display

Selection rounds were carried out independently at three different pH values: 5.5, 5.0 and 4.5. As expected, the number of rounds of selection that could be carried out decreased as the pH value during the selection was lowered. At pH 5.5 seven rounds of binding-elution could be carried out, at pH 4.5 only four. After these rounds of selection from each pH group a number of phagemids were picked and analyzed (Table 3A).

The sequences of the selected phages show some striking features. Only variants in positions 435, 437 and 439 are found after selection, whereas variants in all 7 positions between residues 433 and 439 are present in the input library (Table 2). Obviously, despite the large number of possible substitutions of residues 433–439, only a few changes are accepted and selected for. That this number is indeed limited is also suggested by the finding that two of the mutants (S3 and S5) are independently selected for in two separate experiments at different pH values.

A closer look at the selected mutants indicates that selection of both single and double mutants has occurred, confirming the presence of both kinds of mutations in the library. Apart from mutants also wild type α-amylase phages are selected for at all pH groups at relatively high frequency (Table 3A). A high proportion of these phenotypic wild-type α-amylase phagemids, however, are encoded by a mutant genotype. One representative of the latter group had even two silent mutations. The selection of such variants indicates that the wild type phenotype is strongly selected for and that, despite the limited occurrence of such a nucleotide sequence in the library, these variants are enriched. Thus we conclude that

Table 2
Diversity of the Cβ4 library with respect to nucleotide (nt) and amino acid (aa) sequence

Mutant #	Nucleotide sequence	aa sequence	nt changes	aa changes
1	GGT GGG GaA cAG CGA ATa TAT	GGEQRIY	3	3
2	GGT GGa aCA AAG CGA ATt TAT	GGTKRIY	3	2
3	GtT GGG GCA AAG gaA ATG aAT	VGAKEMN	4	3
4	GGT Gcc GCA AcG tGA Act gAT	GAAT*	7	5
5	Gca GGG cCt AAG CGA tTG cAg	AGPKRLQ	7	4
6	GaT GtG tgg cAG CGA cTa TAT	DVWQRLY	8	5
7	GGT GGG ttA AtG CGg ATG TAT	GGLMRMY	4	2
8	aGT GGt tCA AAG CcA cTG TAT	SGSKPLY	5	4
9	GGT aGG GCA AAt CGA ATG cAT	GRANRMH	3	3
10	GGT GGG GCA AAG CGA tTG TAT	GGAKRLY	1	1
11	Ggc Gct GCA AAG CGg ATG TAT	GAAKRMY	4	1
12	GGT GGG GCA AAG CGA ATG TgT	GGAKRMC	1	1
13	GGT GGG Gac tAG CGA ATG TAT	GGD*	3	2
14	GGT GtG GCc tAc CGt ATG TAc	GVAYRMY	6	2
15	GGT GGG GCA AAG Cct ATG TgT	GGAKPMC	2	2
16	GGT GGG GCA AAG CGA AgG TAT	GGAKRRY	1	1
17	GGT GGG GCA tAG CGA ATG TgT	GGA*	2	2
18	GGT GcG GCA tAG CGA ATG TAT	GVA*	2	2
19	GGT GGt tCA gAt CGA gTG aAT	GGSDRVN	6	4
20	GaT GGG aCA AAG CGA ATG TAT	DGTKRMY	2	2
21	GGT GcG ttG AAG CGA ATG TAT	GALKRMY	3	2
22	GtT GcG atA AAa CGA Act TAT	VAIKRTY	7	4
23	GGT GGG GaA ttG CGA ATG TtT	GGELRMF	4	3
24	GtT GGc tCA cAG atA cTc TAT	VGSQLLY	8	5
25	GcT GGG GCA AAG CcA ATG Ttc	AGAKPMY	4	2
26	GGT GGa GtA tAG aGc ATG TAT	GGV*	5	3
27	Gga GcG GCc AAG aGA ATG TAT	GAAKRMY	4	1
28	Gga GGG GCA AgG Cac ATG cAT	GGARHMH	5	3
29	tGT Gca GCA AtG CGg tTG TAg	CAAMRL*	7	5
30	GGT GaG GCA cAG aGA AaG TAc	GEAQRKY	5	3
31	GGT ccG GCA AAG CGc ATG TAT	GPAKRMY	3	1
32	Gag tGt tCA AAG CcA ATc TAT	ECSKPIY	7	5

Table 3A
Overview of the selected variants

pH	Phage survival (%)	Rounds	Phages left	Sequenced	Mutants (aa/nt)
5.5	50	7	100	8	4/6
5.0	0.5	5	130	8	3/6
4.5	0.5	4	110	6	3/4

the selection procedure is strong and that the wild-type enzyme has a strong starch-binding property. We therefore expect that the (phenotypic) mutant α-amylase phagemids that are selected side by side with the wild-type enzyme have a binding capacity at least similar to the wild type.

3.6. The Cβ4 domain is involved in starch-binding

The library was created to have only amino acid mutations in the Cβ4 domain, which is in a domain distant from the active site residues. Therefore, it can be expected that starch-binding

might be altered whereas the integrity of the catalytic site is not affected. In order to dissect these two processes the binding of the α-amylase to starch and the catalytic activity were analyzed separately by comparing the hydrolysis reaction on its natural substrate (starch) and on an artificial oligosaccharide. For an efficient starch hydrolysis the enzyme has to adhere with its starch-binding domain to the starch granule before the enzyme can scroll along the polymer and therefore an intact starch-binding domain is essential. We have used cross-linked blue-starch (Phadebas) to determine the activity of enzyme variants on starch, which is measured by the formation of the blue coloured agent. The mutants that have been taken from the Cβ4 library without selection show a diverse pattern: some mutants have almost wild type behaviour while other α-amylase variants barely degrade starch. In all cases a decreased reaction on starch correlated with a decreased growth on starch plates of the bacteria harbouring the mutant α-amylase. The mutants that accumulated after selection were found to have an activity similar to the wild type enzyme in this test. We conclude from these data that the Cβ4 domain is indeed important for starch-binding.

In Fig. 3 the performance in the Phadebas test is compared to the activity on the artificial oligosaccharide EPS. The substrate EPS is generally used to measure the turnover number of α-amylases. Hydrolysis yields the yellow coloured nitrophenol and in the presence of α-glucosidase the reaction can be monitored continuously. Mutants with a defective starch-binding domain are expected to give a low ratio of activities in this test, while mutants with a functional starch-binding property yield a high ratio. It can be seen that the randomly picked population contains many mutants with defective starch-binding properties whereas selection only yields mutant (and wild type) enzyme that have proper starch-binding characteristics (Fig. 3). This implies that residues in the Cβ4 domain, which is far from the active site, are essential for good starch-binding. Novel starch-binding mutants can be selected out of the Cβ4 phage library using the 1-day quick select method variants (Table 3B).

3.7. Selected mutants show improved starch-binding at low pH

The activity on starch for the mutant enzymes was determined at two different pH values: pH 7.5 and 4.5. Starting the enzyme reaction at the desired pH and taking aliquots from the reaction mixture at different time intervals accomplished this. In Fig. 4A the specific activities of the selected mutants and the wild type samples are plotted. Bradford analysis was used to correct for the variations in protein yield from the Ni^{2+}-nitrolotriacetate protein preparations. It can be seen that the specific activities of the mutants are in the same order as compared to the wild type α-amylase. This result is in accordance with the data of Fig. 3.

The relative activity of the selected mutants at pH 4.5 and 7.5, respectively, is presented in Fig. 4B. This ratio is independent from protein concentration and can be determined more accurately. The activity ratio of the wild type enzyme at pH 4.5 and 7.5 is approximately 0.050, illus-

Table 3B
Results of the selection procedures

Variant	Amino acid sequence	Number of mutations	pH during selection
WT	433-GGAKRMY-439	0	–
S1	GGAKLMF	2	5.0
S2	GGAKWMY	1	5.0
S3	GGSKRMY	1	4.5 and 5.0
S4	GGPKRMF	2	5.5
S5	GGAKRMF	1	4.5 and 5.5
S6	GGAKCMY	1	5.5

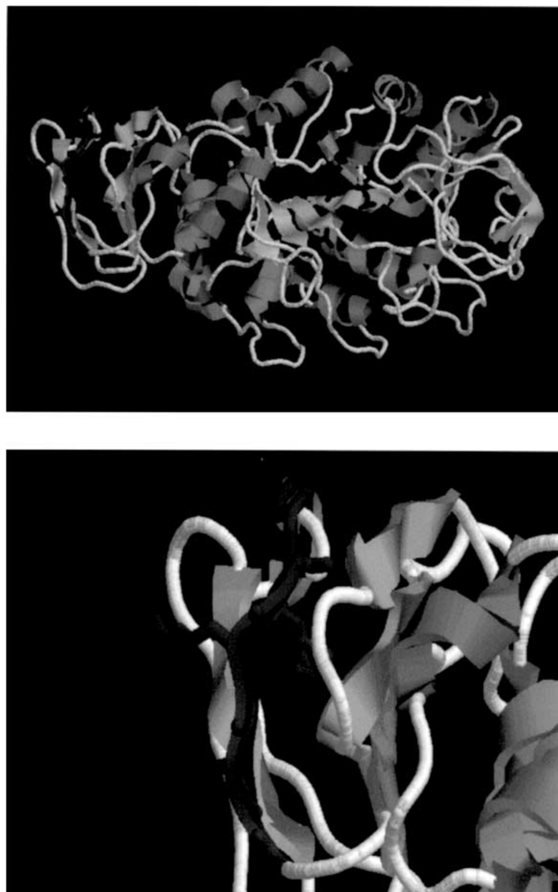

Fig. 2. Region of α-amylase used for selection: (A) General 3D structure of the α-amylase. The active site residues are represented in black. Five β-sheets potentially involved in starch-binding are shown in light grey. The side chains of residues of Cβ4, which were randomized in the phage library, are represented in dark grey; (B) Detail of the starch-binding domain of α-amylase. The five β-sheets potentially involved in starch-binding have been selected based on visual inspection of the 3D structure. The corresponding residues are 402YFDHH406, 433GGAKRMY439, 446GETWHDITGN455, 456RSEPVVI-NSE465, 465EGWGEFHVN473 (10). Color pattern as in A.

trating the decrease in starch hydrolysis of the wild type enzyme when the pH is lowered. This ratio between the activity on pH 4.5 and 7.5 is significantly increased for some of the selected mutants up to 0.130 for variant S4. This indicates that these selected mutants are less affected by the decrease in pH. Therefore we conclude that the starch hydrolysis activities of the selected α-amylase variants S1–S6 at pH 4.5 are improved in comparison to the wild type enzyme.

4. Discussion

Phage display has been used extensively as a tool to select for protein with improved affinities. Although it was suggested that display techniques could also be used to select improved enzymes, the demonstration of a practical example has still to be realized. As a major reason for this failure, it has been put forward that display techniques can only be used to select for binding, not for catalysis (Forrer et al., 1999).

As binding can be considered as an essential part of catalysis we have decided to explore the value of phage display for enzyme engineering using an industrial enzyme that has a limitation in substrate binding. On the basis of the observed difference in activity on complex starch substrates versus simple sugars it was hypothesized that the thermostable α-amylase from *B. licheniformis* has a starch-binding domain (Helbert et al., 1996). The precise annotation of this domain onto the structure was never done, however. On the basis of similarity with CGTases we propose five β-sheet regions (see Fig. 2B) as potential starch-binding domains. We have prepared a library of α-amylase mutants that contains all one and two amino acid changes in the C-terminal 4 domain, which is far apart from the active site residues in the αβ-barrel central domain (Fig. 2A). By randomly picking and sequencing a number of these mutants the diversity of the library was demonstrated and the presence of many halo deficient clones, which still maintained activity on EPS, confirms the involvement of Cβ4 in starch-binding. This very same library of mutants, now expressed as fusions with phage coat protein gp3, was used to select for variants with improved starch-binding properties using surface display. The fusion of gp3 and amylase could be detected by both amylase and gp3 antibodies and it was established that the amylase/gp3 fusion was displayed in active form at the surface of around 5% of the phages. The selection procedure was firstly

designed with model mixtures of amylase and wild type phages. With cross-linked starch, conditions for binding amylase at various pH conditions were established. Under these conditions also the amylase displaying phages were bound selectively to the cross-linked starch, albeit at a rate slower than the soluble enzyme. After leading the mutant phages through several rounds of starch-binding/elution, without intermediate multiplication of the enriched phage fraction, a number of variants were selected from the Cβ4 library. In our opinion, the first round in this selection procedure merely serves as a method to separate the non-α-amylase displaying phage particles from the ones that do display the enzyme. In the following rounds the actual selection occurs. Moreover, the method we used, i.e. starch-binding at low temperatures followed by elution at elevated temperatures, may well necessitate hydrolysis of the starch by the displayed protein to dissociate the phage from the matrix. Based on these data we can not exclude that we have selected our mutants on the basis of both substrate binding followed by substrate hydrolysis.

Evaluation of the library composition in relation to the mutants that retain their starch hydrolytic activity show a preference for an amino acid substitution on residue 435, 437 or 439 (Table 3B). These residues are pointing outward from the enzyme (Fig. 2B), indicating that changes of an amino acid pointing to the outside of the β-sheet are tolerated. Most mutants that have lost their activity are the ones that have altered side chains pointing inward (Table 4).

The mutation Y439F (mutant S5) was selected in two independent experiments during this study and two further mutants (S1 and S4) have the Y439F mutation next to another replacement. Apparently, this change is favorable for the phage enzyme to become selected, even if this is based on a limited number of individuals in this study (Table 3A). Interestingly, BLAST alignment indicates that the tyrosine in that position is replaced by phenylalanine in some naturally occurring amylases including the rumen bacterium *B. circulans* (data not shown). As we can conclude from our data, the reason for this is most likely its enzymatic performance: the mutation does not alter the activity on starch and maltoheptasoide at

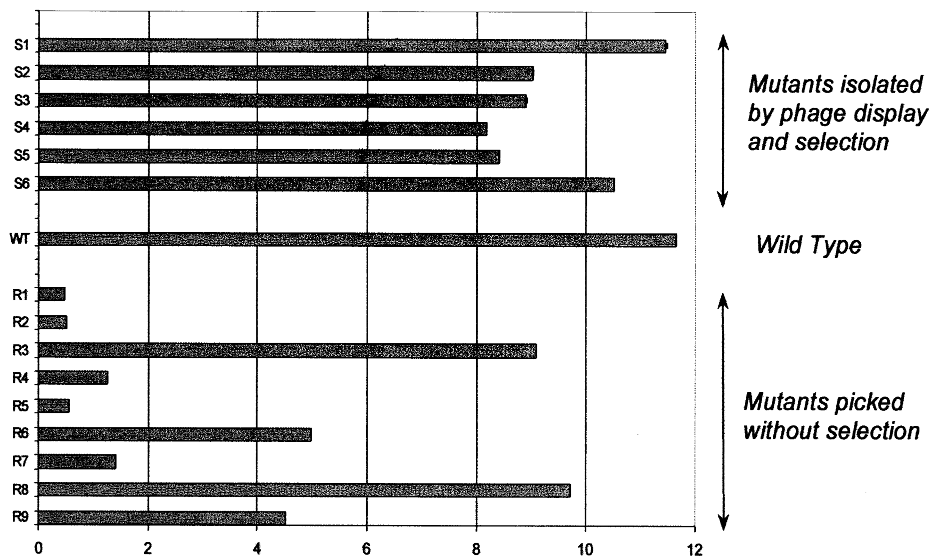

Fig. 3. Relative activity of α-amylase on starch at normal pH (7.5). The activity of periplasmic proteins from *E. coli* cells containing mutant α-amylase on starch, requiring both starch-binding and hydrolysis, in relation to the activity on the heptamaltose EPS, requiring only active site activity, is plotted on the horizontal axis. Mutants S1–S6 have been selected by phage display. Mutants R1–R9 were randomly picked.

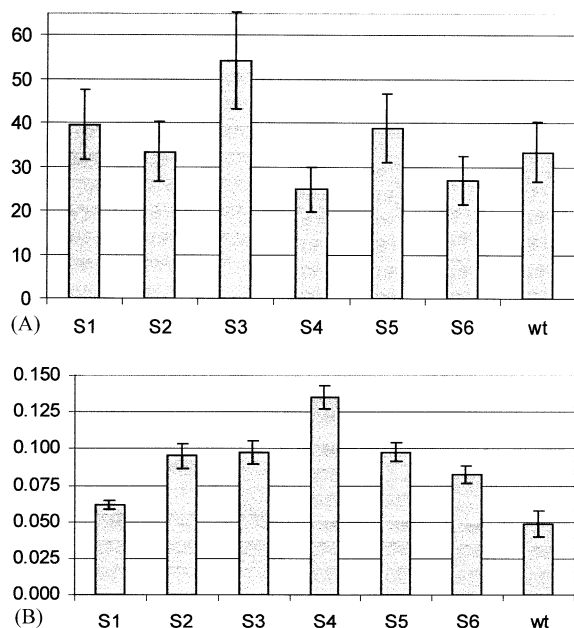

Fig. 4. (A) Specific activities (U mg^{-1}) of the selected mutants as determined on starch (Phadebas) are plotted on the Y-axis. Protein concentrations have been determined by Bradford analysis. Standard errors are given; (B) Comparison of activity of selected mutants at different pH values. The purified periplasmic amylases as described in Fig. 3 were measured for activity on starch (Phadebas) pH 4.5 and 7.5. The ratio between the activity at pH 4.5 and 7.5 is given at the *Y*-axis. Data are based on 8 measurements. The standard error of the means is indicated by the error bars. All mutants have a significantly better ratio compared to wild type amylase.

neutral pH, but the performance of the enzyme at low pH value increases. Also the A435S mutation was selected twice (S3). The characteristics of this mutant are comparable to the previous (S5) mutant both in specific activity as well as in pH behaviour. Apparently increasing the polarity at that amino acid position, without changing the net charge, enables the enzyme to interact better with the starch at low pH values. This could also explain the R437C mutation.

The other mutations, R437L and R437W, replacing arginin at position 437 were found once. This may indicate that the charged arginin is less favorable for the behavior of the enzyme at low pH. The change of this residue into the amphyphilic tryptophan (S2) gives rise to the highest enhancement of activity at low pH. The double mutant S4 (A435P, Y439F) is the most interesting mutant since its relative activity on pH 4.5 is improved most compared to the wild type. The change from a relative structural undefined amino acid to proline, which is known to affect the structure of a protein, is very remarkable. At this moment we have no structural evidence of the result of this change and X-ray diffraction may be necessary to explain the precise effect on starch-binding.

The mutants selected by us are very diverse and only in retrospect we can explain the rationale for their selection. It is hardly feasible that these improvements could have been predicted by rational design and therefore this study demonstrates that phage display is a powerful selection technique for enzymes with improved substrate binding. Interestingly, all mutations in α-amylase reported up to now without exceptions are in the αβ core domain (for review see Nielsen and Borchert, 2000). We expect that surface display not only can be used to address other potential

Table 4
Comparison of selection versus screening mutants isolated by selection

Mutants isolated by selection
G G A K R M Y
A S W H
P S F
L
C
Active mutants isolated by screening for starch degradation (halo formation on starch plate)
G G A K R M Y
A A S M W L H
S V L I F
D P R S C
L L
T C
E P
H
Inactive mutants isolated by screening for starch degradation (small or no halo formation)
E V W Q E I N
R I N R Q
E Y V
C D T
K

starch-binding parts of α-amylases using larger libraries, but it may be also a strong selection technique for other enzymes with a distinct substrate binding step or substrate binding domain.

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