Directed evolution of a bacterial α-amylase: Towards enhanced pH-performance and higher specific activity.

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Summary

α-Amylases, in particular, microbial α-amylases are used widely in industrial processes such as starch liquefaction and pulp processes and more recently in detergency. Following the need for α-amylases adapted to latter, we enhanced the alkali-activity of the α-amylase from *Bacillus amyloliquefaciens* (BAA). The genes coding for the wild type BAA and the mutants BAA S201N and BAA N297D were subjected to error prone PCR and gene shuffling. For the screening of mutants we developed a novel, reliable assay suitable for high throughput screening based on the Phadebas® assay. One mutant (BAA 42) has an optimal activity at pH 7, corresponding to a shift of one pH unit compared to the wild type. BAA 42 is active over a broader pH-range than the wild type resulting in a fivefold higher activity at pH 10. In addition, the activity in periplasmic extracts and the specific activity increased 4 and 1.5 fold, respectively. Another mutant (BAA 29) possesses a wild type like pH-profile but reveals a 40-fold higher activity in periplasmic extracts and a nine fold higher specific activity. The comparison of the amino acid sequences of these two mutants with other homologous microbial α-amylases revealed the mutation of the highly conserved residues W194R, S197P and A230V. In addition, three further mutations were found K406R, N414S and E356D, the latter being present in other bacterial α-amylases.

Key Words: Directed evolution, α-amylase, pH-activity profile, specific activity, high throughput assay

Introduction:

The main natural substrate of α-amylases (EC 3.2.1.1. 1.4-α-D-glucan glucanohydrolases) is starch, which is cleaved into branched and unbranched oligosaccharides. The industrial interest in α-amylases is based on their application in sugar producing processes and more recently in detergency where thermostable α-amylases and α-amylases with high activity are required, respectively. The α-amylase from *Bacillus amyloliquefaciens* (BAA) is a liquefying α-amylase with a temperature optimum of 50-70 °C and a pH optimum of 6. The BAA is stable up to 50 °C and shows 12% residual activity after incubation at pH 12 (Granum 1979). The industrial applications of BAA are starch liquefaction and detergency (Norman 1982; Kottwitz *et al.* 2001). Other liquefying α-amylases frequently used in industrial processes are the α-amylases from *Bacillus licheniformis* (BLA) and *Bacillus stearothermophilus* (BStA), which share an amino acid sequence similarity of 87% and 73% to BAA.

The use of α-amylases in industrial processes raised the need for their adaptation to the given process conditions. As a consequence, protein engineering techniques have been applied to the BLA to improve its thermal stability by rational protein engineering (Svensson and Sogaard 1992; Svensson 1994; Declerck *et al.* 1995). Recently, the tolerance of the BLA towards low pH was enhanced by directed evolution (Shaw *et al.* 1999). Extensive studies were carried out to identify determinants for the pH-profile of BLA (Nielsen *et al.* 1999; Nielsen *et al.* 2001). Comparably few work has been done on improving the BAA, focusing on the thermostability of the BLA. Thermostability determinants were identified by the construction of BAAxBLA hybrids (Conrad *et al.* 1995) or by deletion of two amino acids of the BAA (Suzuki *et al.* 1989). More examples for protein engineering of bacterial α-amylases can be found in (Nielsen and Borchert 2000).

The structure of α-amylases, consisting of the three domains A, B and C is highly conserved. Domain A, which is a $(\alpha/\beta)_8$-barrel (TIM-barrel), includes the N-terminus and the active site.

Domain B shows the highest variability within several structures (Svensson 1994), and is almost exclusively formed by β-strands. Domain C, which forms a Greek key motif is located on the other side of the TIM-barrel and contains the C-terminus.

Directed evolution is a powerful tool to improve protein properties (Kuchner and Arnold 1997) consisting usually of the creation of a pool of mutated genes and the subsequent screening for improved gene products. Improved mutants can be subjected to consecutive rounds of mutagenesis and screening. We used error-prone PCR to randomly introduce mutations to the gene coding for the BAA in combination with gene shuffling (Stemmer 1994 a and b) for the recombination of mutations. For the detection of amylase activity, we developed a high throughput screening protocol based on the activity of the α-amylase toward an insoluble dye-conjugated starch polymer. To screen simultaneously for improved activity at high pH and improved specific activity, the setup of a two dimensional screening was considered to reduce the appearance of expression mutants and other false positives, a problem frequently seen in directed evolution experiments (Bornscheuer *et al.* 1999; Zhao *et al.* 1999). Since an improved activity at alkaline pH was desired, we decided to screen at two different pH-values, pH 7 and pH 10. Wild type clones and wild type like clones as well as improved clones were expected to be located within defined regions of the two dimensional plot (Figure 1): Wild type clones or wild type like clones should lay along an axis with the slope of the wild type ratio of pH 7/pH 10 activity whereas variants with improved activity at pH 10 should have a lower slope. In addition, variants with improved specific activity and/or improved expression/procession properties should have a longer distance to the origin. Variants with both parameters improved should have a lower slope and a longer distance from the origin.

Since the screening at different pH values requires enzyme solutions with low buffer capacity, care has to be taken with the sample preparation. *E. coli* has no mechanism for the active secretion of heterologously expressed proteins into the media. Therefore, disruption of the

cells is needed for the release of expression products into the medium. The BAA expressed in *E. coli* is secreted into the periplasmic space (Pretorius *et al.* 1988), allowing the isolation of the BAA from the periplasm by a cold osmotic shock. Unfortunately, existing protocols require multiple pipetting and incubation steps (Neu and Heppel 1965) and therefore lower the throughput. We decided to facilitate the secretion of the BAA into the culture medium by the coexpression of the Bacteriocin release protein (BRP) (van der Wal *et al.* 1995 a and b;). The BRP stimulates the phospholipase C, which in turn hydrolyses the phospholipids present in the inner and outer cell membrane, thereby generating permeable regions. The secretion of an α-amylase from *E. coli* by BRP coexpression has been demonstrated successfully (Yu and San 1992).

Results:

Expression of the BAA and the coexpression of BRP in *E. coli*

The BAA wild type gene was cloned into the *Nde*I-*Pst*I-site of the plasmid pG-PFE, to give pG-BAA (Figure 2). The expression of BAA in *Escherichia coli* XL1-Blue under control of the rhamnose promoter proved to be constitutive, since uninduced cultures showed substantial amounts of α-amylase activity. Nevertheless, the coexpression of the Bacteriocin Release Protein (BRP) from plasmid pJL3 for the secretion of the BAA increased the α-amylase activity in the culture medium accompanied by a strong increase of the α-amylase activity in periplasmic fractions (Figure 3).

Assay evaluation

The microtiter plate modified Phadebas® HTS assay for the determination of α-amylase activity was evaluated by measuring different dilutions of the commercial α-amylase BAN 240L in a 96 well microtiter plate with 8 replicas of each activity. Between 0 and 1000 U/L a linear correlation between absorption and activity was found with R^2=0.9995. The linearity is considerably high for activities up to 2000 U/L (Figure 4). The relative standard deviation ranges from 0.8% to 5.6%. The average relative error lies between 1.7% and 11.8% (Table 1). For screening of mutant libraries, the assay was set up as a two dimensional screening at pH 10 and pH 7. To determine the background of the assay, a population of 96 α-amylase negative clones (*Escherichia coli* XL1-Blue cotransformed with the plasmids pG-PFE coding for an esterase from *Pseudomonas fluorescence* and pJL3 encoding BRP to allow secretion) were induced and screened at pH 7 and pH 10 as described in the methods section. The absorption values at pH 7 and pH 10 were plotted against each other resulting in a background of about 0.15 absorption units in each dimension. In order to determine the wild type region 96 colonies of *E. coli* transformed with the plasmids for BAA and BRP expression (*E. coli*

(pGBAA WT, pJL3))were induced and screened. Due to the distribution of the measured values, the wild type area was defined to lie between the curves A(620. pH 7)= 8xA(620. pH 7) and A(620. pH 7)= 8xA(620. pH 7)-0.8 (Figure 5).

Starting points for directed evolution

In addition to the wild type BAA we used the two point mutants BAA S201N and BAA N297D as starting points for directed evolution. Both mutants were constructed by site directed mutagenesis of the BAA gene (Bessler *et al.* 2000). The activity of BAA S201N at pH 10 and pH 11 is increased by 16% and 50%. While the activity of BAA N297D at pH 10 is comparable to the wild type, the activity at pH 11 is increased by 50%.

Construction and screening of the error-prone library

A random library of BAA mutants was created under the conditions described resulting in the production of 7200 clones. Screening for α-amylase active colonies was performed on agar plates containing 1% of starch and activity staining with iodine. The inactivation rate was found to be 30%. DNA-sequencing of 40000 base pairs of randomly picked clones revealed a mutation rate of 0.6/1000 corresponding to 1 mutation per gene for the 1545 bp gene. Screening for α-amylase activity at pH 10 and pH 7 using the modified Phadebas® HTS assay identified 26 clones with improved activity compared to the wild type. DNA-sequencing proved 10 of these to be wild type or possessing silent mutations, while 16 carried at least one non-silent mutation.

Construction and screening of the DNA-shuffling library

The BAA genes of these 16 clones were subjected to DNA-shuffling resulting in a total of 10000 clones. 960 active clones were found by activity staining as described, corresponding to a deactivation rate of 90 %. Screening of these 960 clones (Figure 5) and subsequent

rescreening as well as screening of retransformed colonies yielded the mutants BAA 42 and BAA 29 which were found to have improved activity at pH 10 (BAA 42) and an improved overall activity (BAA 29 and BAA 42). Both mutants were sequenced completely and further characterized.

Characterization of the mutants: Biochemical analysis

Both BAA 42 and BAA 29 show an enhanced activity in periplasmic fractions due to a higher protein concentration and a higher specific activity (Table 2). The activity concentration b in periplasmic fractions of BAA 42 is increased by a factor of about 3.6. In periplasmic fractions of BAA 29, the activity concentration is about 41.2 times higher. While the specific activity of BAA 42 is enhanced 1.5 fold, the specific activity of BAA 29 is more than 9.3 times higher compared to the wild type. To determine if the mutants show higher expression rates the approximate α-amylase concentration in periplasmic extracts was calculated from the ratio of activity concentration b over z/m. For BAA 42, the value is 2.4 times higher than for the wild type, for BAA 29 even 4.5 times.

The pH activity profile (Figure 6) of BAA 42 shows an optimum at pH 7 compared to the wild type, with an activity optimum at pH 6. The relative activities of BAA 42 at pH 9 and 10 are about 1.5 and 5.7 times higher than those of the wild type resulting in a broader pH activity profile for BAA 42. In contrast, the pH activity profile of BAA 29 is very similar to the wild type profile with an pH-optimum of pH 6 and only a marginally lower relative activity at alkaline pH.

Characterization of the mutants: Sequence analysis

DNA-sequencing of BAA 42 revealed five mutations (L13P, W194R, S197P, E356D and N414S) leading to a change in the amino acid sequence. Three of these mutations, W194R, E356D and N414S were inherited from the error-prone PCR derived clones BAA 18, BAA 19

and BAA 1, respectively (Figure 7). The mutation L13P and the mutation S197P were acquired during the process of recombination. The mutation L13P is located in the signal peptide of the α-amylase. The mutations W194R, S197P, E356D and N414S lie within the mature α-amylase. Comparison with the amino acid sequences of other bacterial α-amylases, namely the α-amylases of *Bacillus licheniformis* (BLA), *Bacillus megaterium* (BMA), *Bacillus* sp. KSM-1376 (LAMY), *Bacillus* sp. #707 (S707), *Bacillus stearothermophilus* (BstA) and *Bacillus* sp. TS-23 (TS-23) shows (Figure 8), that the mutations W194R and S197P are located in a highly conserved region. In fact W194 is present in every sequence of the compared α-amylases. In the case of S197 only BMA possesses a glycine at this position. Both mutations are located on a solvent accessible loop in domain B of the BLA structure (Figure 9).

At position 356, a glutamate is present in the sequences of BAA and BMA whereas in all other amylase sequences this position is occupied by an aspartate. Therefore, the mutation E356D represents a mutation towards a higher conserved amino acid. The amino acid 356 is located in the core of domain A of the α-amylase structure, being not accessible to the solvent. At position 414, the asparagine is replaced by serine in mutants BAA42 and BAA 29. In the sequences of BLA, BMA, LAMY, S707, BstA, and TS-23, a conserved lysine is found at this position which is located on the surface of the structure in one of the TIM-barrel forming α-helices of domain A.

In BAA 29, six amino acids were replaced: L13P, V32A, N297D, K406R, and N414S. From these, V32A, K406R and N414S were inherited from the clones BAA 13, BAA 4, and BAA 1. N297D was adopted from BAA N297D over clone BAA 3. The mutations L13P and A230V were generated during the shuffling process. In addition to the L13P mutation in the signal peptide, mutation V32A is located at the N-terminus of the mature protein. V32A

converts the VNG-sequence into ANG, which is known to be processed by the *E. coli* signal peptidase with higher probability according to the von Heijne rule (von Heijne 1985).

In the mature α-amylase, BAA 29 contains the mutations A230V, N297D, K406R and N414S, with the latter also being present in BAA 42. A230V is located on a β-strand in the B domain above the active site of the α-amylase, on the surface of the structure. Amino acid comparisons show that A230 is conserved in the amino acid sequences of the α-amylases BAA, BLA, BMA, LAMY, S707, BStA, and TS-23. Mutation K406R is also located in domain A, on the top of a loop, and is accessible to the solvent. Mutation N297D which is located in an α-helix of domain A was found to stabilize the local structure by a salt bridge (Bessler *et al.* 2000).

Discussion:

To our knowledge this paper is the first on the successful improvement of the alkali activity of an α-amylase by directed evolution. Though some efforts have been done on changing the pH-profile of the BLA by site directed mutagenesis (Nielsen *et al.* 1999; Nielsen *et al.* 2001), only one example is known to literature, where the activity of the BLA for acidic conditions has been increased (Shaw *et al.* 1999). The specific activity of the BLA has so far been only improved by site directed mutagenesis (Nielsen *et al.* 1999; Nielsen *et al.* 2001).

We could achieve a high α-amylase activity in the periplasm and the effective secretion of the BAA in the medium by coexpression of BRP in *E. coli* XL1-Blue cells. This method also sped up sample preparation from 90 minutes to about 60 minutes, reducing the needed pipetting steps from 4 to1 and thus increasing the throughput. Together with the Phadebas® assay modified for the use in microtiter plates, we developed a versatile assay system for α-amylases with a very low error and standard deviation. The variability of the experimental

conditions allows the adaptation to a multitude of parameters. For example, by altering the reaction temperature, screening can be performed to search for thermophilic or cold-active α-amylases. The stability of the substrate also allows screening in the presence of detergents and other chemicals present in industrial applications.

The finding of false positives, i.e. the identification of wild type genes as positive clones, in screening is a common problem. This is mainly due to the complexity of both the expression system and the enzyme containing solution. It is known to literature, that although cultivated in the same microtiter plate under the same conditions, expression can differ significantly from well to well (Zhao *et al.* 1999).

On the other hand, by setting up our assay in two dimensions and defining a sharp region for wild type BAA, we sucessfully identified positive mutants. The method of measuring several parameters and correlating the results has been successfully applied to other screening methods before (Ness *et al.* 1999; Morawski *et al.* 2000; Wintrode *et al.* 2000). Nevertheless, it seems obligatory to perform rescreening of retransformed colonies in order to validate screening results.

The inactivation rate of 30% and a mutation rate of 1/gene was sufficient to successfully generate improved mutants by error-prone PCR, although present theoretic investigations suggest, that higher deactivation rates and therefore higher mutation rates provide better access to a higher diversity of improved mutants (Miura and Sonigo 2001).

The DNA shuffling of the BAA mutants obtained by error prone PCR resulted in two mutants with strongly altered properties. However, the inactivation rate of almost 90 % required the subsequent selection of active clones for keeping the screening costs at a low level. In the case of amylases this can be easily done by a pre-screening using the starch agar plate assay/KJ plates.

Both mutant BAA 42 and BAA 29 carry mutations that were inherited from clones created by error-prone PCR as well as mutations created during DNA shuffling. Mutant L13P shows

that mutations are introduced at any step of the DNA shuffling as it is not found in any error-prone clone but in both mutants. Therefore, it had to be introduced during an early step of the shuffling process.

It is quite difficult to predict the influence of every single mutation found in BAA 29 and BAA 42. Although not proven, it is likely that the amino acid substitutions found in both mutants (L13P, N414S), are not responsible for a change in the pH activity profile by themselves. Hence, the mutations W194R, S197P and E356D of BAA 42 seem to be responsible for the change in the alkaliphilicity. They may lead only in combination with N414S to the change in the pH activity profile. Furthermore, since both mutant proteins are found in higher concentrations in the periplasm than the wild type, L13P seems to improve the processing.

Looking at the structure of BAA, it is surprising, that the mutations were found in the domains A and B but not in domain C. Therefore it seems, that domain C is more vulnerable towards negative mutations than domain A or B.

None of the mutations found in BAA 42 or BAA 29 were described earlier to have an influence on the pH activity profile or the specific activity (Nielsen *et al.* 1999; Nielsen *et al.* 2001).

Conclusions

The methods used in this paper show the possibility of an efficient trimming of the BAA towards multiple parameters required for industrial applications. The assay used in this experiment allows to screen for several parameters at once, e.g. thermal activity and thermal stability as well as solvent and salt depending parameters. In addition, it can be applied to every α-amylase containing solution in a pH-range of pH 4 to pH 12. We were able to alter the pH activity profile resulting in a shift the pH-optimum by one pH unit to pH 7. In addition, we improved the specific activity of the BAA by the factor 1.5 (BAA 42) and 9.3

(BAA 29) as well as the expression and processing of the BAA. The comparison of the amino acid sequences of the mutants with other homologous α-amylases showed that mutations in highly conserved regions not necessarily lead to inactivation. By combination of mutagenesis and recombination with a multi dimensional assay, several parameters can be improved in a few steps. Further rounds of mutagenesis and recombination can be used to further improve the BAA.

Materials and methods

Materials

All chemicals used were of reagent grade or higher. As long as not other specified, chemicals were received from Sigma Chemie (Deisenhofen, Germany). Oligonucleotides were obtained from Sigma-Ark (Darmstadt, Germany). Restriction enzymes, T4-DNA ligase and desoxynucleotides were purchased from MBI Fermentas (St. Leon-Rot, Germany). MAR5N50 filter microtiter plates and Centricons® were obtained from Millipore (Eschborn, Germany).

Cloning of the BAA gene in pGPFE

The BAA gene in the cloning vector pGEM-Teasy was a gift of Henkel KGaA, Düsseldorf. Plasmid pG-PFE encoding an esterase from *Pseudomonas fluorescence* under control of the rhamnose promoter was kindly provided by Erik Henke (Henke et al. unpublished results). A 1700 bp fragment containing the BAA gene (1545 bp) was cloned after *Bam*HI-*Spe*I-digestion into the expression vector pCYTEX P1 cut with the same enzymes to give pCYTBAA using standard procedures (Sambrook *et al.* 1989). After introduction of a *Nde*I-site at the start codon of the BAA gene by site directed mutagenesis (Dalbadie-McFarland *et al.* 1982) using *Pfu*-Polymerase (Stratagene, La Jolla, USA) and the primers 5'-GAGAGGGAGAGGACATATGATTCCAAAACG-3' and 5'-CGTTTTGGAATCATATGTCCTCTCCCTCTC-3', the resulting plasmid pCYTBAA-NDE was *Nde*I-*Pst*I-digested and the BAA containing DNA fragment was ligated into the *Nde*I-*Pst*I-site of pG-PFE giving pG-BAA. The correct orientation of the BAA gene and the correct sequence were determined by DNA-sequencing.

Random mutagenesis and recombination

Libraries of BAA mutants were constructed using error prone PCR and plasmid pG-BAA as described (Zhao *et al.* 1999): 10 µl 10x mutagenic buffer (70 mM $MgCl_2$, 500 mM KCl, 100 mM Tris pH 8, 0.1 (w/v) gelatin), 10 µl 10x mutagenic dNTP-mix (2 mM dGTP and dATP, 20 mM dCTP and dTTP), 50 pmol each of the primers BAF 5'-CTTAAGAAGGAGATATACATATG-3' and BAR 5'-GCCAAAACAGAAGCTTGGCTGCAG-3', 5-15 ng of template, 60 µM $MnCl_2$ and 5 U *Taq* polymerase (Qiagen, Hilden, Germany) in a total volume of 100 µl. Thermal cycling parameters were: 1 min 95 °C; 30 s, 95 °C, 45 s, 55 °C, 30 s, 72 °C (30 cycles); 2 min 72 °C. The amplified product was purified using the QIaQuick PCR-Purification kit (Qiagen, Hilden, Germany), digested with *Nde*I and *Pst*I and ligated into the *Nde*I-*Pst*I-site of the dephosphorylated vector pG-PFE. For expression of the BAA variants, *E. coli* XL1-blue competent cells (Stratagene, La Jolla, USA) were at first transformed with pJL3 (MoBi-Tech, Göttingen, Germany), a plasmid encoding the Bacteriocin release protein (BRP) gene using the protocols suggested by the manufacturer to give *E. coli* XL1-blue (pJL3). Subsequently, competent *E. coli* XL1-blue (pJL3) cells were transformed with the variants of the pG-BAA plasmid.

DNA Shuffling

For DNA shuffling, the plasmid variants of pG-BAA from the error prone PCR were isolated from positive clones and the BAA genes were amplified by using the primers 5'-GCAAAAACAGGAAGGCAAAATGCCG-3' and 5'-CCTCCGGGCCGTTGCTTCGCAACG-3' in a PCR reaction containing 5-10 ng of template, 3 mM $MgCl_2$, 50 pmol of each primer, 0.2 mM of each dNTP and 2.5 U of *Taq* polymerase in a total volume of 100 µl of the 1x buffer supplied by the manufacturer. The temperature

program was: 240 s, 95 °C; 60 s, 95 °C, 60 s, 55 °C, 60 s, 72 °C (25 cycles); 240 s, 72 °C. The products were purified with the Wizard PCR Preps-Kit (Promega, Mannheim, Germany) and subjected individually to partial DNaseI digestion for 7 min at 15 °C using 0.01 mU DNaseI (Boehringer now Roche Diagnostics, Mannheim, Germany) in 25 mM Tris/HCl (pH 7.4) in the presence of 5 mM $MnCl_2$ in a total volume of 50 µl. Fragments of 50-200 bp of the different templates were extracted from 1% agarose gels using the QIaExII kit (Qiagen, Hilden, Germany), and mixed in equimolar amounts. For the reassembly, a PCR-reaction without primers containing 10 µl of fragment mix, 3 mM $MgCl_2$ and 2.5 U of *Taq* polymerase in a total volume of 50 µl was performed. PCR program: 90 s, 95 °C; 30 s, 95 °C; 30 s, 60 °C; 30 s, 55 °C; 60 s+5 s/cycle (35 cycles), 72 °C; 300 s, 72 °C. The full length product was amplified from the reassembly mixture using the primers BAF and BAR under the same conditions as the first PCR and cloned into pG-PFE as described above. Clones producing active α-amylase were pre-screened by plating on LB-Amp-agar plates containing 1% of soluble starch, and staining with a saturated aqueous solution of I_2/KI for 10 s. Positive clones were identified by the formation of a clear halo against the violet background.

Microtiter plate assay

Colonies from LB-agar plates with ampicillin and chloramphenicol were isolated by a picking robot (Biorobotics, Cambridge, UK) into 96 well microtiter plates containing 150 µl LB supplemented with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol ($LB_{amp,\ cm}$) and were grown at 37 °C for 16 h. After this period, 50 µl 50% sterile glycerol were added to each well and the microtiter plates were stored at –80 ºC. For expression cultivation, microtiter plates containing 200 µl $LB_{amp,\ cm}$ supplemented with 0.2% rhamnose and 5 µM IPTG were inoculated from the glycerol stocks and cultivated for 42 hours at 37 °C in a fully equilibrated

incubator. One freeze thaw cycle (-196 °C, 5 min; 37 °C, 60 min; 0 °C, 15 min) followed by centrifugation (910 xg, 15 min, 15 °C), yielded the enzyme containing supernatant.

Screening for increased alkali activity was carried out by measuring the activity at pH 10 and pH 7. For measurements at pH 10, 4 Phadebas® tablets (Pharmacia and Upjohn, Erlangen, Deutschland) were suspended in 30 ml double distilled water and filtered over a fritted glass filter (pore size 0 or 1). The retentate was resuspended in 32 ml 0.1 M glycine/NaOH buffer (pH 10). For measurements at standard pH (pH 7), 4 Phadebas® tablets were suspended in 32 ml double distilled water. 150 µl of the well mixed suspension of the appropriate pH were added to each well of a MAR5N50 filter microtiter plate by means of a 8 channel multipette. 50 µl of enzyme solution were added to each well using a Biomek 2000 (Beckman Coulter, Unterschleissheim-Lohhof, Germany) pipetting robot. After incubation for 15 min at 37 °C in a fully equilibrated incubator and centrifugation at 910 xg for 15 min at 15 °C, the absorption of the flow through was measured at a wavelength of 620 nm in a Fluorstar microtiter plate reader (BMG Labtechnologies, Offenburg, Germany).

The standard curve at pH 7 was obtained by measuring the α-amylase activity of different dilutions of the commercial α-amylase BAN240L, which was kindly provided by Novo Nordisk (Mainz, Germany).

Cuvette assay

α-amylase activity was routinely measured using a modified protocol of the Phadebas® assay. 1 tablet was suspended in 10 ml double distilled water. 500 µl of the resulting suspension were transferred to a microfuge tube , 50 µl of the appropriately diluted enzyme (< 1000 U/l) were added and incubated for 15 min at 37 °C. The reaction was stopped with 150 µl 0.5 M NaOH and the remaining substrate was removed by centrifugation (20000xg, 15 min). 500 µl of the supernatant were mixed with 500 µl of double distilled water in a micro cuvette before

the absorption (620 nm) was measured. pH-Activity profiles from pH 4 to pH 11 were performed using the corresponding 50 mM buffer (pH 4-6 acetate, pH 7-8 Tris/HCl, pH 9-10 glycine/NaOH and pH 11 carbonate) and appropriately diluted α-amylase containing solution (<1000 U/L).

Isolation from periplasm and protein purification

For the isolation of the recombinant α-amylase, 50 ml $LB_{amp,\ cm}$ medium were inoculated 1:100 from an overnight culture and incubated at 37 °C and 200 rpm until OD_{600}=0.4. Protein expression was induced with 0.2% rhamnose. Cells were harvested 3 hours after induction by centrifugation (3200 xg, 15 min, 15 °C) and the pellets were subjected to a cold osmotic shock in order to release the proteins from the periplasm (Neu and Heppel 1965).

The purification was done by adsorption of the α-amylase to starch (Candussio *et al.* 1990) and each step was carried out at 4 °C (including solutions and instruments). Periplasmic fractions were mixed 1:10 with 0.1 M glycine/NaOH buffer pH 9. After the precipitate was removed by centrifugation, the supernatant was brought to a final concentration of 0.1 M Tris/HCl (pH 7), 20 mM $CaCl_2$ and 20% saturation of $(NH_4)_2SO_4$. Soluble potato starch was added to a final concentration of 3% and adsorption was performed in an over head shaker (30 rpm, 360°) for 3 hours. The pellet was removed by centrifugation (3300 xg, 15 min, 4°C) and the supernatant was treated with 3% soluble potato starch for another 1 h. The pellets were combined and washed with ½ of the initial volume of a 0.1 M Tris/HCl buffer pH 7.5 containing 20 % saturated $(NH_4)_2SO_4$ and 1 M NaCl. Desorption from the starch was achieved by treating the pellets with ½ of the initial volume of a Tris/HCl buffer (0.1 M, pH 7.5) containing 3 M NaCl and 0.1 M maltose for 90 min. After centrifugation (3300 xg, 15 min, 4 °C) the pellet was again treated using the same buffer. The supernatants were combined,

concentrated 10 fold using a Centricon® (30 kDa cutoff) and desalted using a PD-10 column (Amersham Pharmacia, Freiburg, Deutschland).

The homogeneity of the enzyme solution was checked by SDS-PAGE according to Laemmli (1970). For storage, 0.025% Brij 35 and 20 mM $CaCl_2$ were added to the enzyme preparation. Protein concentration was determined using a commercial BCA-assay (Pierce Chemical Company, Rockford, Illinois) using BSA as standard.

Computer modelling methods

The theoretical structure of BAA was obtained by homology modeling from the SwissModel server (Peitsch *et al.* 1995; Peitsch 1996; Peitsch *et al.* 1996; Guex and Peitsch 1997) with the structure of BLA (1bli.pdb) as template, which was received from the protein databank (PDB) (Bernstein *et al.* 1977). Amino acid mutations were inserted to the structure using the mutate tool of the SwissPDB-Viewer, followed by the sidechain reconstruction for neighbored amino acids and energy minimization.

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In memoriam of Douglas Adams – the authors disbelieve, that 42 is the answer to the question.

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Figure legends

Figure 1. The two dimensional screening system. α-Amylase activity was measured at pH 10 and pH 7. Clones with wild type like pH activity profile should be located between the parallels, whereas clones with improved activity at alkaline pH should appear to be shifted rightwards. Clones with improved activity should have a longer distance from the origin.

Figure 2. Map of plasmid pGBAA for expression of BAA in *E. coli*. Expression is under control of the rhamnose inducible promoter P_{Rh}. The plasmid contains an ampicillin resistance gene Amp^R, and a pUC18 origin of replication.

Figure 3. Expression of BAA in *E. coli* XL1-Blue. For induced cultures, 0.2% rhamnose were added at OD_{600}=0.4. For both rhamnose induced and uninduced cultures, the expression of BRP was induced with 20 μM IPTG one hour after OD_{600}=0.4 was reached. Cultivation was stopped 3 hours after IPTG induction, periplasmic extracts were prepared, and the α-amylase activity in the supernatant and the periplasmic extracts was determined.

Figure 4. Calibration curve of the modified Phadebas® HTS assay. The linear range lies between 0 U/L and 1000 U/L. The error bars show the maximal errors of 8 replicates within one row of a microtiter plate.

Figure 5. Screening of the DNA-shuffling library. ◇ DNA-shuffling library, □ best hits from the same library, △ best hits from the error-prone library. The two mutants BAA 29 and BAA 42 are annotated.

Figure 6. pH activity profiles. Activity measurement was carried out using the following 50 mM buffers: pH 4-6 acetate, pH 7-8 Tris/HCl, pH 9-10 glycine NaOH, pH 11 carbonate. □ WT, × BAA 29, ○ BAA 42

Figure 7. Phylogenetic tree of BAA 29 and BAA 42. New mutations of each round are annotated by an asterisk.

Figure 8. Parts of a ClustalX sequence alignment of BAA, BAA 29 and BAA 42 with the α-amylases from *B. licheniformis* (Sibakov and Palva 1984; Stephens *et al.* 1984), *B. megaterium* (BMA) (Metz *et al.* 1988), *B.* sp KSM-1376 (LAMY)(Igarashi *et al.* 1998), *B.* sp #707 (S707)(Tsukamoto *et al.* 1988), *B. stearothermophilus* (BStA) (Nakajima *et al.* 1985), *B.* sp. TS-23 (TS-23)(Lin *et al.* 1998). Mutated amino acids are printed in bold face.

Figure 9. Mutations in the structure of BAA. The structure was yielded by homology modeling based on the structure of BLA (1bli) using the SwissModel server (http://www.expasy.ch/swissmod).

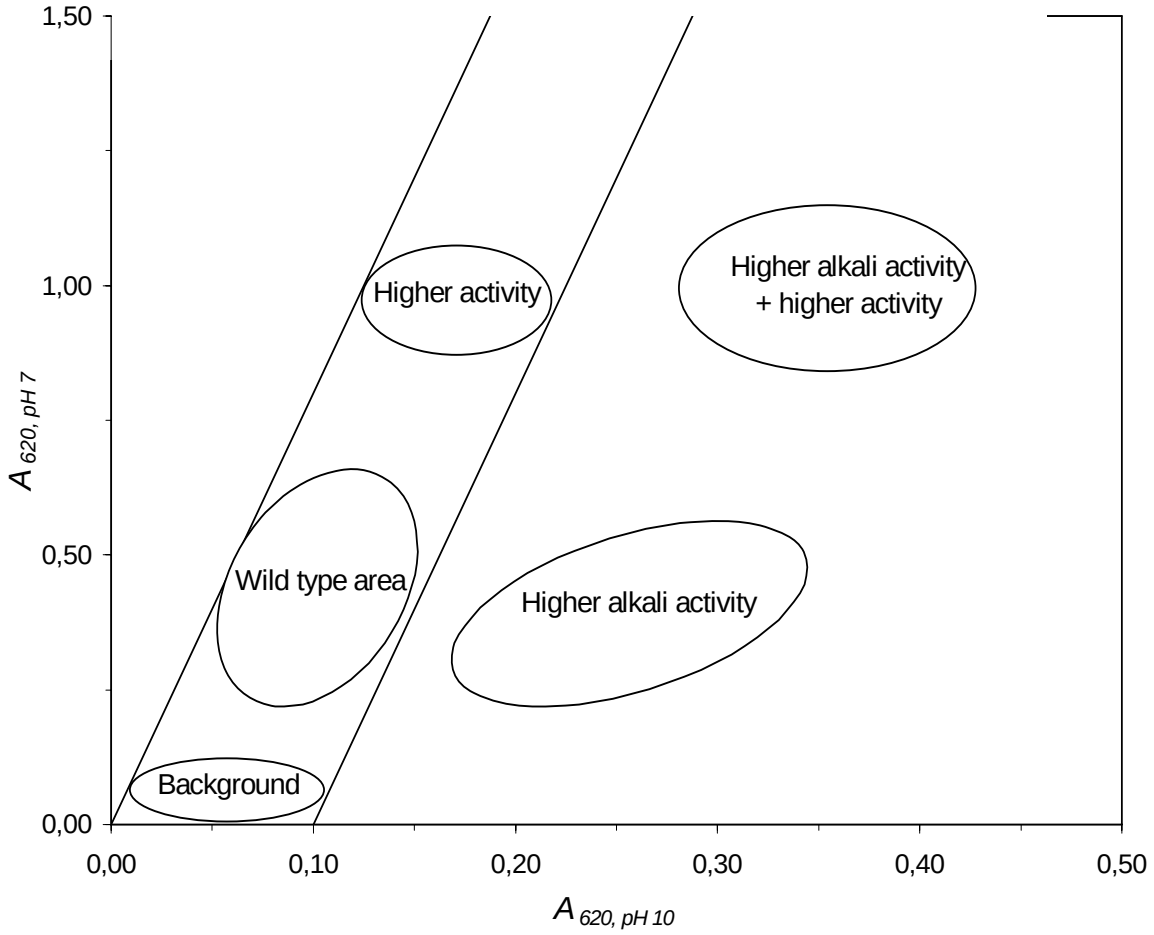
1,50
1,00
0,50
0,00
0,00
0,10
0,20
0,30
0,40
0,50
A 620, pH 7
A 620, pH 10
Higher activity
Higher alkali activity
+ higher activity
Wild type area
Higher alkali activity
Background

Figure 1

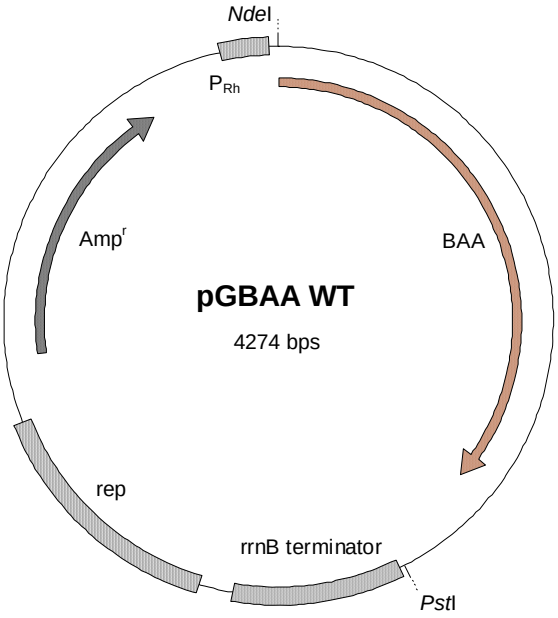

Figure 2

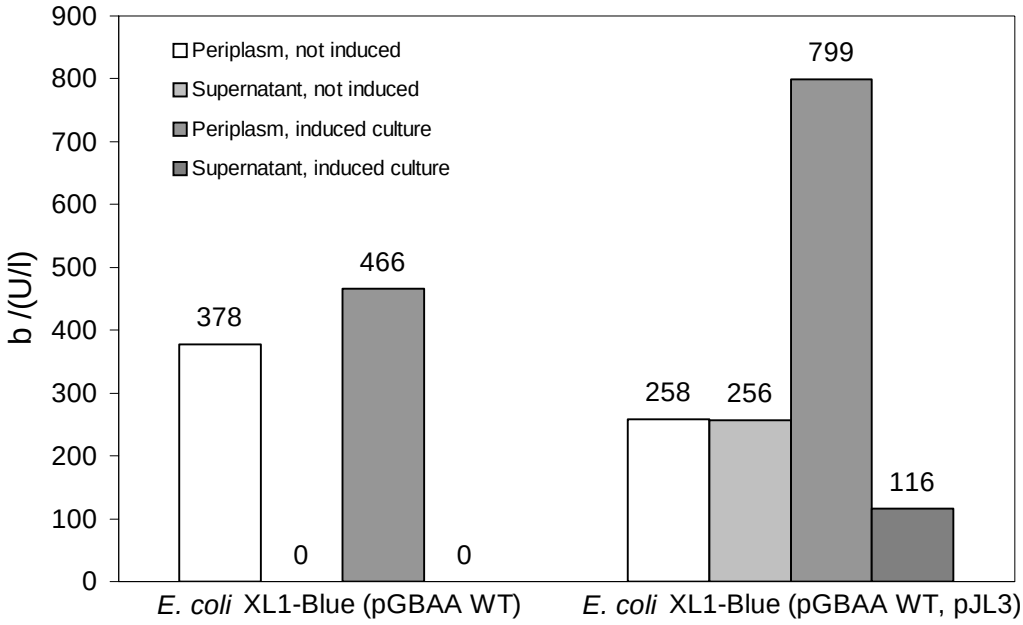
900
800
700
600
500
400
300
200
100
0
b /(U/l)
Periplasm, not induced
Supernatant, not induced
Periplasm, induced culture
Supernatant, induced culture
378
0
466
0
258
256
799
116
E. coli XL1-Blue (pGBAA WT)
E. coli XL1-Blue (pGBAA WT, pJL3)

Figure 3

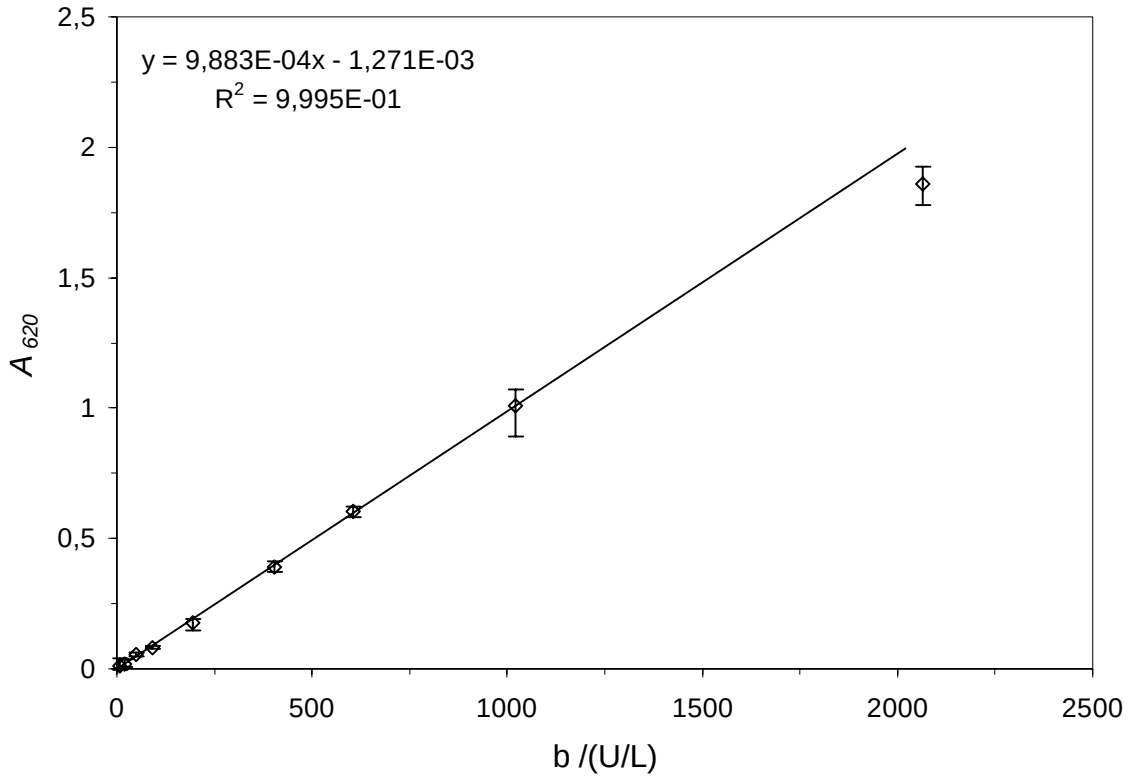
y = 9,883E-04x - 1,271E-03
R2 = 9,995E-01
A 620
b /(U/L)
2,5
2
1,5
1
0,5
0
0
500
1000
1500
2000
2500

Figure 4

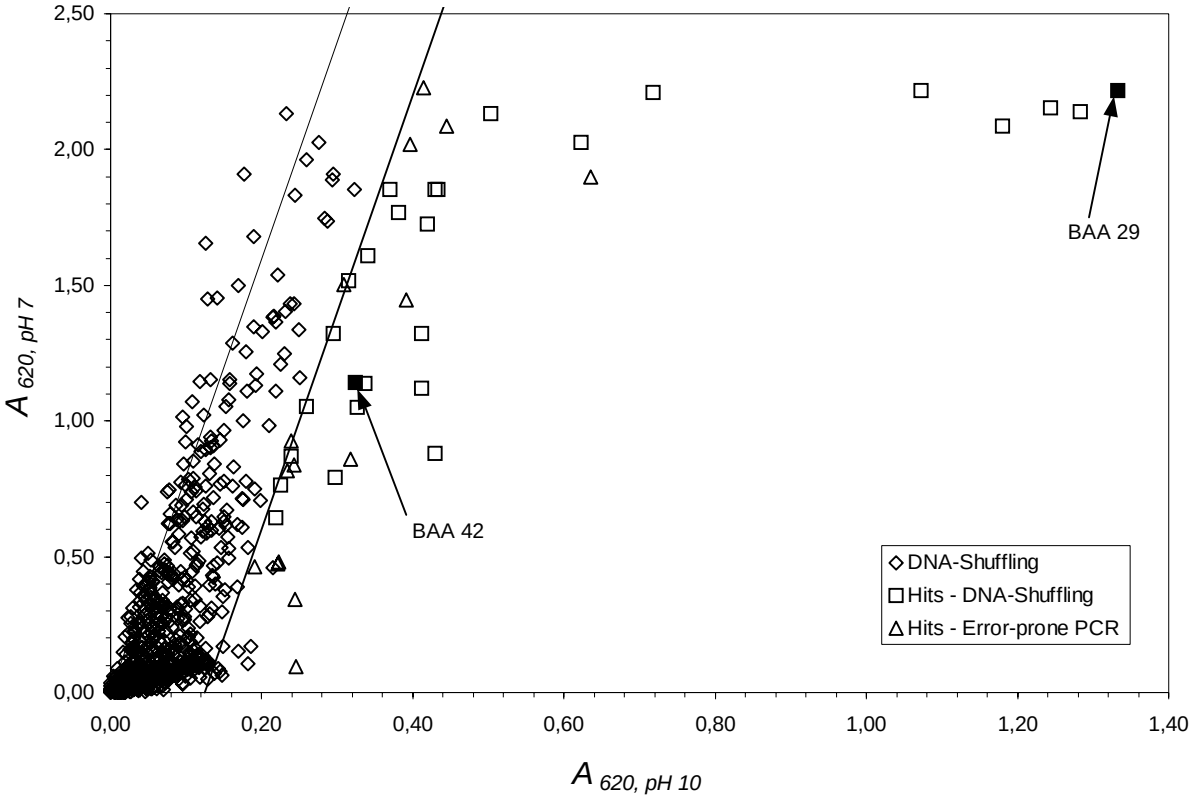
2,50
2,00
1,50
1,00
0,50
0,00
0,00
0,20
0,40
0,60
0,80
1,00
1,20
1,40
A 620, pH 7
A 620, pH 10
BAA 29
BAA 42
DNA-Shuffling
Hits - DNA-Shuffling
Hits - Error-prone PCR

Figure 5

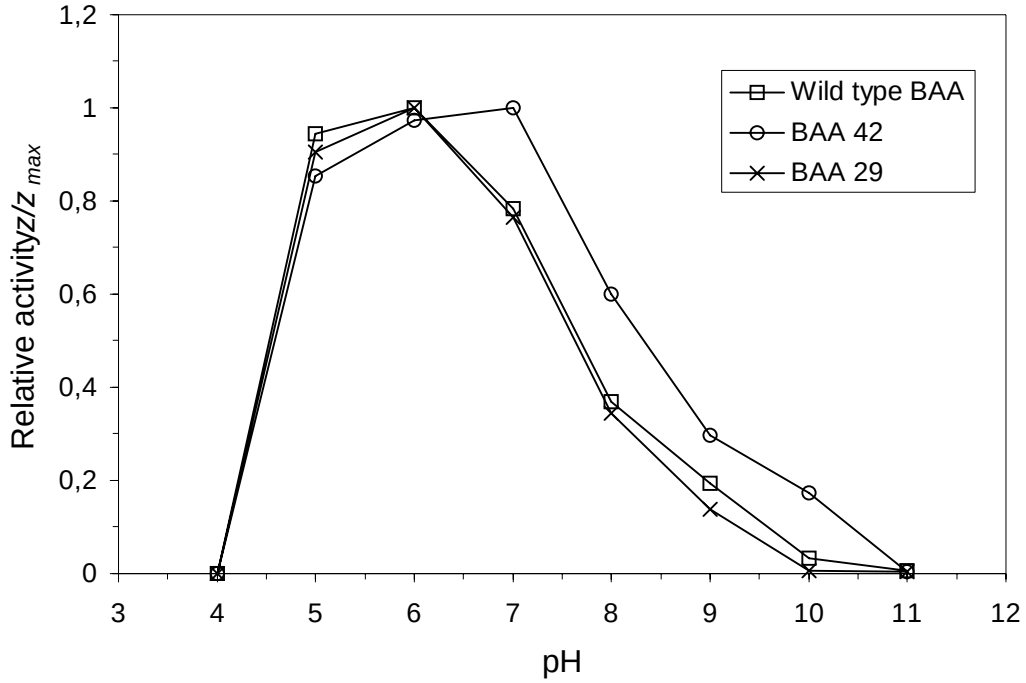
1,2
1
0,8
0,6
0,4
0,2
0
Relative activityz/z max
3
4
5
6
7
8
9
10
11
12
pH
Wild type BAA
BAA 42
BAA 29

Figure 6

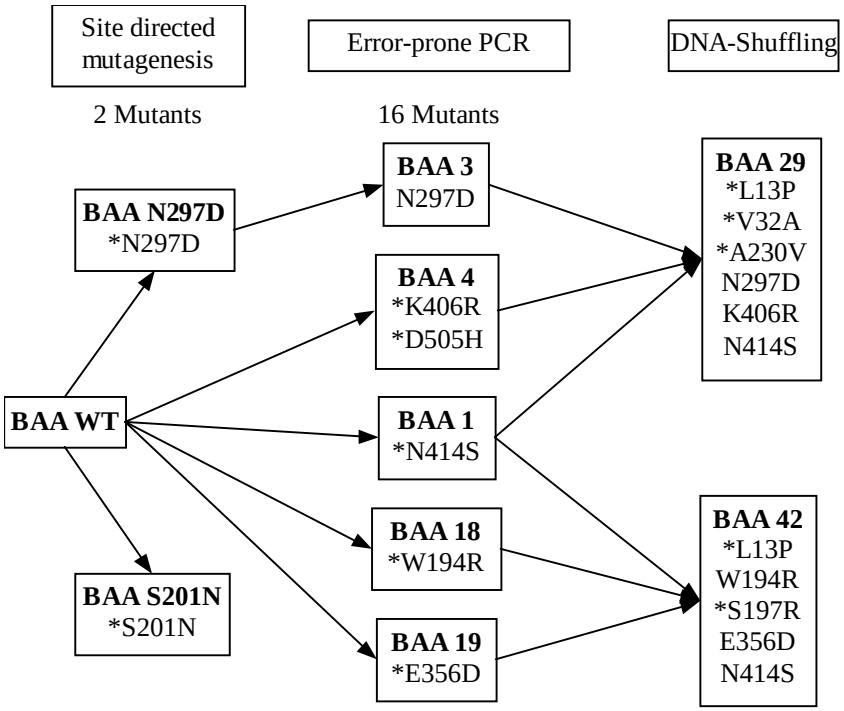
Site directed mutagenesis
Error-prone PCR
DNA-Shuffling
2 Mutants
16 Mutants
BAA N297D
*N297D
BAA WT
BAA S201N
*S201N
BAA 3
N297D
BAA 4
*K406R
*D505H
BAA 1
*N414S
BAA 18
*W194R
BAA 19
*E356D
BAA 29
*L13P
*V32A
*A230V
N297D
K406R
N414S
BAA 42
*L13P
W194R
*S197R
E356D
N414S

Figure 7

```
                13          31          194197
                 |           |            |  |
BAA 29      TVSFRPVLMCT…TKTSAANGTLM…FDGADWDESRKIS-R…
BAA 42      TVSFRPVLMCT…TKTSAVNGTLM…FDGADRDEPRKIS-R…
BAA         TVSFRLVLMCT…TKTSAVNGTLM…FDGADWDESRKIS-R…
BLI         YARLLTLLFAL…AAAANLNGTLM…FDGTDWDESRKLN-R…
BMA         IVGLSVVMFLP…YADTVNNGTLM…FDGTDWDEGRKLN-R…
LAMY        LTLLLAVAVLF…AHHNGTNGTMM…FDGTDWDQSRQLQNK…
S707        LAFLLVITS-I…AHHNGTNGTMM…FDGVDWDQSRRLNNR…
BStA        FLLAFLLTALL…KAAAPFNGTMM…FDGVDWDESRKLS-R…
TS-23       YSIIATLVISF…ANTAPINETMM…FDGTDWDESRKLN-R…
Consensus      L       …A     NGTMM…FDGTDWDESRKLN-R…

                230         356          406     414
                 |           |            |       |
BAA 29      DYLMYVDVDYD…AVTFVENHDTQ…KGTSPREIPSLKDSIEPIL
BAA 42      DYLMYADVDYD…AVTFVDNHDTQ…KGTSPKEIPSLKDSIEPIL
BAA         DYLMYADVDYD…AVTFVENHDTQ…KGTSPKEIPSLKDNIEPIL
BLI         DYLMYADIDYD…AVTFVDNHDTQ…KGDSQREIPALKHKIEPIL
BMA         DYLMYADLDFD…AVTLVENHDSQ…KGNSNYEIPALKDKIDPIL
LAMY        DYLMYADIDMD…AVTFVDNHDSQ…PTHG---VPSMKSKIDPLL
S707        DYLMYADIDMD…AVTFVDNHDSQ…PTHG---VPAMRSKIDPIL
BStA        DYLMYADLDMD…AVTFVDNHDTE…PQYN---IPSLKSKIDPLL
TS-23       DYLMFADLDMD…AVTLVDNHDTQ…PKYN---IPGLKSKIDPLL
Consensus   DYLMYAD DMD…AVTFVDNHDTQ…P   ---IP LKSKIDPIL
```

Figure 8

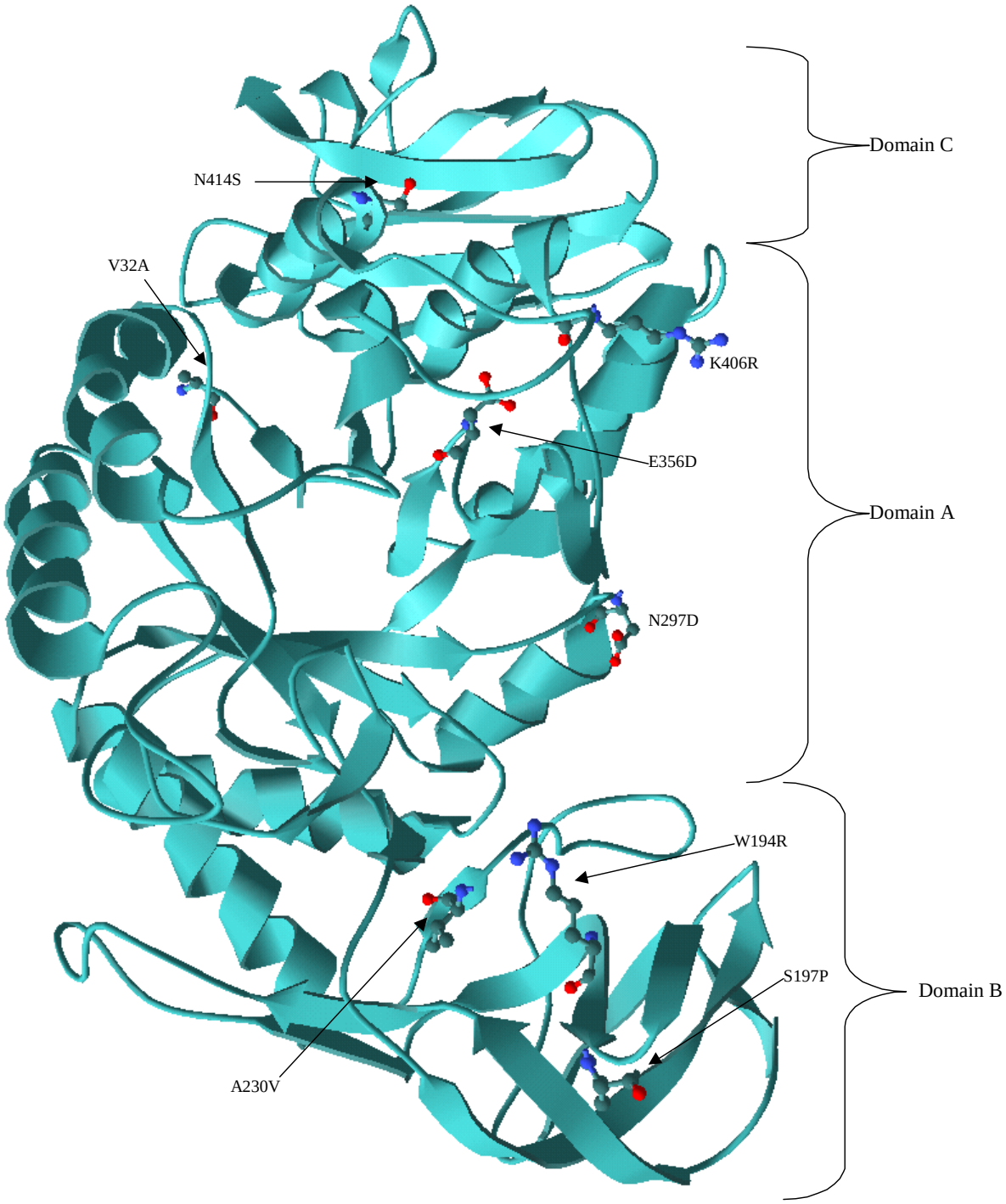
Domain C
N414S
V32A
K406R
E356D
Domain A
N297D
W194R
S197P
Domain B
A230V

Figure 9

Tables

Table 1: The modified Phadebas® Assay. The standard curve was obtained by measuring the α-amylase activity of a dilution row of BAN 240L at pH 7.

b /(U/l)	A(620)	Standard deviation	Avg. error	Avg. rel. error/%
0	0.000	0.008	0.012	6.8
8	0.012	0.013	0.022	11.8
19	0.020	0.008	0.014	7.3
49	0.055	0.004	0.006	2.8
91	0.082	0.003	0.004	1.7
195	0.178	0.013	0.022	6.3
403	0.392	0.013	0.022	3.8
605	0.604	0.014	0.021	2.7
1021	1.007	0.056	0.091	7.7

Table 2: Activity and specific activity of BAA, BAA 42, and BAA 29. The values in brackets give the relative activities and concentrations in comparison to the wild type.

	b (periplasm) [U/L]	Specific activity z/m [U/mg]	Protein concentration (periplasm) [mg/L]
BAA WT	1420±38 (1.00±0.03)	15.1±1.1 (1.00±0.07)	93.8 (1.0)
BAA 29	59484±1602 (41.89±1.13)	140.9±9.5 (9,27±0.63)	422.2 (4.5)
BAA 42	5035±95 (3.55±0.06)	22.0±1.2 (1.46±0.08)	229.0 (2.4)

Footnotes

Abbreviations

BAA: α-amylase from *Bacillus amyloliquefaciens*

BLA: α-amylase from *Bacillus licheniformis*

BStA: α-amylase from *Bacillus stearothermophilus*

LAMY: α-amylase from *Bacillus* sp. KSM-1376

S707: α-amylase from *Bacillus strain #707*

TS-23: α-amylase from *Bacillus* sp. TS-23

E. coli: *Escherichia coli*

BRP: Bacteriocin release protein