Combinatorial engineering to enhance amylosucrase performance: construction, selection, and screening of variant libraries for increased activity

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Abstract **Amylosucrase is a glucosyltransferase belonging to family 13 of glycoside hydrolases and catalyses the formation of an amylose-type polymer from sucrose. Its potential use as an industrial tool for the synthesis or the modification of polysaccharides, however, is limited by its low catalytic efficiency on sucrose alone, its low stability, and its side reactions resulting in sucrose isomer formation. Therefore, combinatorial engineering of the enzyme through random mutagenesis, gene shuffling, and selective screening (directed evolution) was started, in order to generate more efficient variants of the enzyme. A convenient zero background expression cloning strategy was developed. Mutant gene libraries were generated by error-prone polymerase chain reaction (PCR), using *Taq* polymerase with unbalanced dNTPs or Mutazyme™, followed by recombination of the PCR products by DNA shuffling. A selection method was developed to allow only the growth of amylosucrase active clones on solid mineral medium containing sucrose as the sole carbon source. Automated protocols were designed to screen amylosucrase activity from mini-cultures using dinitrosalicylic acid staining of reducing sugars and iodine staining of amylose-like polymer. A pilot experiment using the described mutagenesis, selection, and screening methods yielded two variants with significantly increased activity (five-fold under the screening conditions). Sequence analysis of these variants revealed mutations in amino acid residues which would not be considered for rational design of improved amylosucrase variants. A method for the characterisation of amylosucrase action on sucrose, consisting of accurate measurement of glucose and fructose concentrations, was introduced. This allows discrimination between hydrolysis and transglucosylation, enabling a more detailed comparison between wild-type and mutant enzymes.**

Key words: Amylosucrase; Zero background expression cloning; Error-prone polymerase chain reaction; Gene shuffling; Positive selection; Efficiency screening

1. Introduction

Amylosucrase is a glucansucrase, a class of enzymes producing glucose polymers using sucrose as the sole substrate [1,2]. Among the glucansucrases the enzyme is unique, since it produces an amylose-like glucan consisting of only α-1,4-linked glucose residues [3]. Furthermore, it is the only glucansucrase belonging to family 13 of glycoside hydrolases, the family including α-amylases, whereas the other glucansucrases constitute family 70 [4]. Contrary to other enzymes responsible for the synthesis of amylose-like polymers [5], amylosucrase does not require the addition of any expensive activated sugar like ADP- or UDP-glucose [6]. Moreover, this enzyme can be used to modify the structure of polysaccharides by the addition of α-1,4-linked glucosyl units [2]. These properties give amylosucrase numerous potential industrial applications. Amylosucrase from *Neisseria polysaccharea* is the only amylosucrase which has been studied as a recombinant enzyme. The gene encoding it has been cloned and expressed in *Escherichia coli*. Methods for production and purification of the recombinant enzyme have been described [2], and its 3-D structure has been solved [7]. It is the best characterised glucansucrase [2,3,8,9], and rational engineering based on structures of amylosucrase complexed with substrate and products [10,11] made it possible to elucidate the structural features implicated in the polymerisation reaction and in the specificity for sucrose [12–14]. Despite the interesting features of amylosucrase from *N. polysaccharea*, its use as an industrial tool for the synthesis or the modification of polysaccharides is limited by its low catalytic efficiency on sucrose alone ($k_{cat} = 1\ s^{-1}$), its low stability ($t_{1/2} = 21$ h at 30°C), and the catalysis of non-desired side reactions resulting in sucrose isomer formation, limiting the yield of insoluble polymer to 45%. Rational engineering recently led to the isolation of a variant amylosucrase with reduced side reactions, increasing the polymer yield to 85% [14]. Specific activity and thermostability, however, are more complex features and are difficult to engineer rationally. These features are usually connected to loop flexibilities, which can be affected by a number of factors such as salt or disulfide bridges, prolines in turns, and hydrogen bonding interactions. Rational engineering of increased thermostability generally involves comparisons of mesophilic enzymes with thermophilic relatives [15]. For increased activity comparisons of more and less active enzymes would be necessary. However, for amylosucrase no such comparisons are possible, and thus more and faster results can be obtained using random techniques such as error-prone polymerase chain reaction (PCR) [16] and gene shuffling [17]. To adapt amylosucrase

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catalytic properties to industrial synthesis conditions, we started a combinatorial engineering project, which aims to combine rational engineering with the use of directed evolution techniques. Here we describe the development of a zero background expression cloning strategy for the generation of large variant libraries, a selection mechanism to discard inactive variants, and a screening method for identification of interesting clones. The properties of two improved variants, isolated using these simple yet effective techniques, are described.

2. Materials and methods

2.1. Bacterial strains and plasmids/growth conditions

One Shot® *E. coli* TOP10 (Invitrogen) was used for transformation of ligation mixtures. *E. coli* JM109 (Promega) was used for screening of amylosucrase variants and large-scale production of the selected mutants. Plasmid pZErO-2 (Invitrogen) was used for subcloning of PCR products and screening, plasmid pGEX-6P-3 (Amersham Pharmacia Biotech) was used for production of glutathione *S*-transferase (GST)–amylosucrase fusion proteins. Bacterial cells were grown on LB (agar) containing 50 μg/ml kanamycin (when harbouring pZErO-derived plasmid), or 100 μg/ml ampicillin (when harbouring pGEX-6P-3-derived plasmid). For expression of amylosucrase in *E. coli* JM109 media were supplemented with isopropyl-β-D-thiogalactose (IPTG) (1 mM). When appropriate, LB agar plates contained 50 g/l sucrose for visualisation of amylosucrase activity, by iodine staining of the amylose-like polymer precipitated in the agar.

2.2. DNA manipulations

Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. DNA purification was performed using QIAQuick (PCR purification and gel extraction) and QIASpin (miniprep) (Qiagen). DNA sequencing was carried out using the dideoxy chain termination procedure [18], by Genome Express (Grenoble, France).

2.3. Cloning and expression of the amylosucrase gene in pZErO

The amylosucrase gene was amplified by PCR with *Pfu* DNA polymerase using ASpUC19 [2] as template and the following primers: ForASpZErO (forward): 5′-CGCAGCAAGCTTG**TAATG**AATTC-ACAGTACCTCAAAAC-3′; RevASpZErO (reverse): 5′-GGTTCA-GACGGCACTCGAGAAGCGTGCG**TCA**GG-3′. The forward primer contains a *Hin*dIII restriction site (AAGCTT) for cloning in pZErO and an *Eco*RI site (GAATTC), which allows cloning of positive variants in the expression vector pGEX-6P-3. The overlapping stop codon (in frame with the *Lac*Z gene) and start codon (in frame with the amylosucrase gene) (TAATG) should lead to expression of amylosucrase [19]. The reverse primer contains a *Xho*I site (CTCGAG) for cloning in pZErO and pGEX-6P-3, and the amylosucrase stop codon (TCA), to make sure that no reading through to the lethal gene encoded on pZErO is possible. The resulting PCR product was digested with *Hin*dIII and *Xho*I and ligated with pZErO digested with the same enzymes. This construct (pCEASE01) was transformed to *E. coli* TOP10 cells and plated on LB agar plates containing sucrose to check amylosucrase activity.

2.4. Generation of variant libraries

Error-prone PCR was chosen as the method to introduce random mutations. Two enzymes, Mutazyme (Stratagene) and *Taq* DNA polymerase (New England Biolabs), were chosen for this purpose, since they are supposed to show complementary bias in mutations introduced. Two new primers were designed, with the same restriction sites and stop and start codons as the ones used for subcloning in pZErO, but perfectly matching pCEASE01: forward: 5′-TATGCAT-CAAGCTTG**TAATG**AATTC-3′; reverse: 5′-GAAGCTCGAG**TCA**-GGCGATTTCGAG-3′. The optimal conditions for error-prone PCR using Mutazyme were investigated by varying the amount of template used in the reaction (from 0.2 to 2 ng/100 μl). The correct conditions for error-prone PCR using *Taq* DNA polymerase were investigated by varying the amount of additional dATP and dGTP (from 0 to 1 mM) and $MnCl_2$ (from 0 to 5 mM), the presence of unbalanced dNTPs and addition of Mn being described as mutagenic factors [16]. The PCR products were digested with *Hin*dIII and *Xho*I and ligated with pZErO digested with the same enzymes. The resulting constructs were transformed to *E. coli* TOP10 cells and plated on LB agar plates containing sucrose. Expression of active amylosucrase was checked by exposing these plates to iodine vapour, allowing evaluation of the amount of inactive variants and the variation in activity of the active clones (variation in intensity and colour after iodine staining of the colonies indicating variation in enzyme activity and/or expression). Variant libraries were constructed using the optimal conditions for both enzymes in 100 μl PCRs, divided into 10 μl aliquots to prevent domination of initial point mutations in the final product.

2.5. Shuffling of the variant libraries

DNA shuffling was performed using the protocol described by Zhao and Arnold [20], based on the shuffling method described by Stemmer [17]. This consists of fragmentation of the genes, a PCR1 without primers to reassemble the genes, and a PCR2 with primers for amplification of the full-length shuffled genes. The only modification of the protocol was made in the fragmentation of the genes; instead of using DNase I, this was performed with restriction enzymes, a method previously described by Kikuchi et al. [21]. 5 μl of the different PCR products (wild-type (W), Mutazyme (M), *Taq* (T)) were incubated with (mixtures of) restriction enzymes for 1 h in a total volume of 10 μl. The reaction mixtures contained *Alu*I and *Ava*I (I), *Fnu*4H (II), or *Hinc*II and *Nae*I (III), each mixture yielding different fragments of 50–400 bp (Fig. 1). This resulted in nine different pools of fragmented genes: WI, wild-type fragmented with *Alu*I and *Ava*I; WII, wild-type fragmented with *Fnu*4H; WIII, wild-type fragmented with *Hinc*II and *Nae*I; MI, Mutazyme product fragmented with *Alu*I and *Ava*I; MII, Mutazyme product fragmented with *Fnu*4H; MIII, Mutazyme product fragmented with *Hinc*II and *Nae*I; TI, *Taq* product fragmented with *Alu*I and *Ava*I; TII, *Taq* product fragmented with *Fnu*4H; TIII, *Taq* product fragmented with *Hinc*II and *Nae*I. Restriction enzymes were inactivated (20 min, 80°C) and 5 μl of the fragmented genes were used in different combinations in 50 μl PCRs without primers according to the following schedule: (A) WI/MII/TIII; (B) WI/TII/MIII; (C) MI/WII/TIII; (D) MI/TII/WIII; (E) TI/WII/MIII; (F) TI/MII/WIII. In the second PCR, using the same forward and reverse primers as used for construction of the initial libraries (see above), 5 μl of the products of the above PCRs were used as template in 100 μl PCRs. The resulting PCR products were digested with *Hin*dIII and *Xho*I and ligated with pZErO digested with the same enzymes. The resulting constructs were transformed to *E. coli* TOP10 cells and plated on LB agar plates containing sucrose. The colonies were scraped from these plates for isolation of the plasmids, constituting the shuffling libraries. Subsequently, the plates were exposed to iodine vapour to determine the amounts of active variants in these libraries (the produced polymers precipitate in the agar).

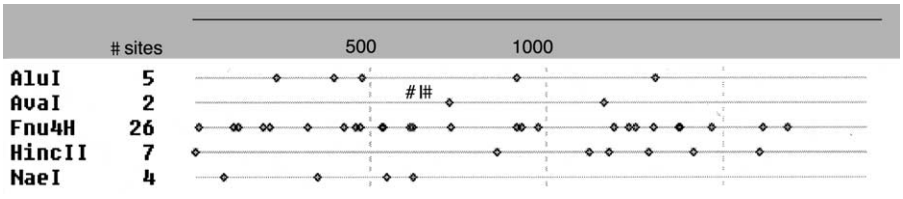

Fig. 1. Restriction map of the amylosucrase gene for the enzymes used for fragmentation of the PCR products.

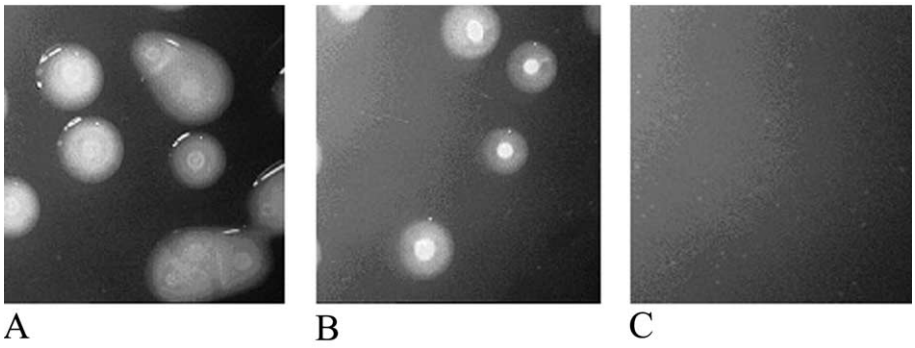

Fig. 2. Selection of amylosucrase active clones on solid mineral medium containing sucrose as the sole carbon source. A: Wild-type. B: 25% active mutant. C: Inactive mutant.

2.6. Selection of active amylosucrase-expressing clones

The shuffling libraries were transformed to *E. coli* JM109 and plated on a 22×22 cm plate containing solid LB, supplemented with 1 mM IPTG and 50 μg/ml kanamycin. After growth the cells were scraped and resuspended and diluted in physiological water to an OD_{600} of 10^{-3}. The clones were then subjected to selection pressure by plating them onto selective solid medium (300 μl of the cell suspension onto a 22×22 cm plate containing solid mineral medium containing 50 g/l sucrose as sole carbon source, 1 mM IPTG, and 50 μg/ml kanamycin). The plates were incubated for 5 days at 30°C, to obtain sufficient cell growth and expression of active amylosucrase.

2.7. Screening of amylosucrase activity

Positive clones were picked using the automated QpixII (Genetix) and transferred to 96 well microtitre plates containing 250 μl LB per well, supplemented with 1 mM IPTG and 50 μg/ml kanamycin. The mini-cultures were horizontally shaken at 250 rpm, for 15 h at 30°C. As amylosucrase is produced intracellularly, the cells were frozen at −20°C preceding the activity determination, in order to increase the sensitivity of the tests and to decrease fructose re-consumption by living cells during enzymatic assays. After de-freezing for 30 min at room temperature, 100 μl of the mini-cultures were transferred into a new microtitre plate, and the OD_{600} was read, to standardise the screening results with the cell growth. Enzymatic reactions were performed using sucrose and glycogen (G-8751, Sigma) (final concentrations 146 mM and 5 g/l, respectively) as substrates, glycogen being a strong activator of amylosucrase activity [8]. After shaking at 250 rpm for 4 h at 30°C, reducing sugar production was measured by adding 50 μl of the reaction mixture to 50 μl of dinitrosalicylic acid (DNS) reagent [22], incubating at 95°C for 7 min, adding 60 μl of this mixture to 180 μl H_2O, and measuring the OD at 540 nm. The formation of the amylose-type polymer was checked by adding 10 μl of iodine solution (100 mM KI, 6 mM I_2, 0.02 M HCl) to the 50 μl of remaining reaction mixture, and measuring the OD at 550 nm, the iodine forming a blue complex with the helical form of amylose [23].

2.8. Production and purification of improved variants

Selected clones were grown in 4 ml LB cultures for plasmid isolation. After sequencing, the genes were subcloned in vector pGEX-6P-3, using the *Eco*RI and *Xho*I restriction sites, for GST fusion protein expression. Production and purification of the variant GST-AS were carried out as described previously [2]. The purity of the enzymes was analysed by electrophoresis on the PHAST system (Amersham Pharmacia Biotech), using PhastGel™ gradient 8–25 (Pharmacia Biotech) under denaturing conditions, followed by staining with 0.5% (w/v) $AgNO_3$.

2.9. Protein concentration determination

Protein concentrations were determined with the Bradford method [24] using the Bio-Rad reagent (Bio-Rad Laboratories) and bovine serum albumin as a standard.

2.10. Biochemical characterisation of improved variants

All assays were performed in 50 mM Tris–HCl buffer (pH 7.0) at 30°C.

Enzyme activity was measured under standard conditions (146 mM sucrose, 0.1 g/l glycogen [2]) using the DNS method [21] to quantify the liberated reducing sugar, using fructose as a reference. One unit of activity was defined as the amount of enzyme producing 1 μmol of reducing sugar per minute.

Kinetic parameters of the action on sucrose were determined by incubating various substrate concentrations (5 mM–1 M) with ∼0.1 mg/ml of pure GST fusion enzyme. At regular time intervals (5 min) 20 μl samples were taken and the amylosucrase was immediately inactivated by heating (3 min, 90°C). After all the samples were taken, the amounts of glucose and fructose formed were analysed using the D-glucose/D-fructose UV method (10 139 106 035, Boehringer Mannheim/R-Biopharm) according to the manufacturer's procedure, but scaled down to be used in microtitre plates.

Polymer formation was analysed by iodine staining of a sample taken after 24 h incubation; the structure and amount of produced polymer was judged by the optimal wavelength and absorption, respectively [23].

3. Results and discussion

3.1. Cloning and selection of (active) amylosucrase gene

A zero background expression cloning strategy of PCR products was developed, using pZErO as vector, to prevent growth of clones without insert, and utilising specific primers, to use the expression from the *lac* promoter on pZErO. *E. coli* cells harbouring this construct indeed express amylosucrase, as shown by their ability to grow on selective medium (see Fig. 2). Non-recombinant clones or recombinant clones expressing inactive amylosucrase do not grow on such a me-

Table 1
Optimal conditions for (error-prone) PCR as determined for different DNA polymerases

	Pfu DNA polymerase (non-mutagenic)	Mutazyme	*Taq* DNA polymerase
$MgCl_2$			1 mM
dNTP	0.2 mM each	0.2 mM each	0.2 mM dTTP/CTP 0.4 mM dATP/GTP
Primers (For and Rev)	0.2 μM each	0.2 μM each	0.2 μM each
Template (ng/100 μl)	2	0.5	2
Enzyme (U/100 μl)	5	2.5	10

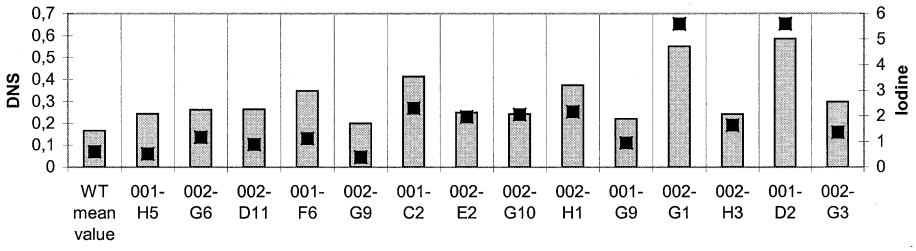

Fig. 3. Screening results of the positive clones using DNS and iodine staining; grey bars indicate DNS response, black squares indicate iodine response.

dium. Furthermore, the size of the colonies was found to be roughly related to the efficiency of the expressed amylosucrase (Fig. 2).

3.2. Generation of mutant libraries

The optimal conditions for error-prone PCR are listed in Table 1. Error-prone PCR using Mutazyme resulted in 70% inactive variants with a nice variation in the iodine staining of the active clones. Error-prone PCR conditions using *Taq* DNA polymerase appear to be a lot less mutagenic than those described to be optimal [16], but result in a sufficient amount of mutations due to the length of the gene. Iodine staining showed nice variation of the active clones and 10% of inactive mutants. Because of the rather high amount of inactive mutants with Mutazyme, we decided to do a shuffling step before screening, to separate possible combinations of positive and negative mutations. Fragmentation of the genes and PCR without primers on mixtures of these different restriction reactions yielded in two cases, for reaction mixtures E and F, a 2 kb band corresponding to the size of the amylosucrase gene. These two products were amplified in a second PCR, subcloned in pZErO, and transformed to yield about 25 000 clones each. Scraping the colonies from the agar plates and plasmid isolation yielded the shuffling gene libraries, plasmids pCEASE01S01E (S01E) and pCEASE01S01F (S01F).

3.3. Selection and screening of positive clones

The shuffling gene libraries (S01E and S01F) were transformed to *E. coli* JM109 and plated on LB agar containing sucrose, to allow the determination of the total amount of transformants and the fraction of active clones. After scraping of the colonies the plates were exposed to iodine vapour to stain the amylose-type polymer that had precipitated in the agar, revealing that less than 5% of the clones were active. The scraped cells were plated on solid mineral medium containing sucrose as the sole carbon source for selection. From these selective plates 60 active clones were picked to inoculate mini-cultures for screening of amylosucrase efficiency. Although most clones showed less activity than the wild-type, the DNS response of 14 clones was at least equal to that of the wild-type amylosucrase. Among these, at least two-fold increased iodine response was observed for eight clones, two of which showed a more than 10-fold increase (clones 002-G1 and 001-D2) (Fig. 3). The ratio between iodine response and DNS response was about three-fold increased for both variants, suggesting that either the yield of incorporation of glucosyl units into the polymer or the length of α-1,4 chains is increased, favouring the complex formation with iodine.

Table 2
Nucleotide substitutions and resulting amino acid replacements of the improved variants

	Nucleotide	Amino acid	Position
001-D2	c123t	Pro41	
	g1165c	Val389Leu	in strand β7
	a1509t	Asn503Ile	in loop 8
002-G1	c58t	Arg20Cys	N-terminus
	g444t	Ala148	
	t1793c	Phe598Ser	C-terminus

3.4. Sequence analysis of improved variants

Sequencing of the two improved clones revealed the mutations listed in Table 2. Both contained three point mutations, in both cases resulting in two amino acid substitutions. Analysis of the structure (Fig. 4) revealed that for 001-D2 the mutations are relatively close to the catalytic site: Val389Leu at the start of β-strand 7, and Asn503Ile in the loop following β-strand 8. However, no direct effect of the mutations can be deduced from the structure, since both residues are relatively far removed from residues that have been shown to be important for the amylosucrase activity [10,14]. For variant 002-G1 the mutations are as far from the active site as possible, Arg20Cys in the N-terminal and Phe598Ser in the C-terminal domain. Furthermore, they are not involved in the secondary binding sites found on the enzyme surface [11]. Obtaining such mutants underlines the importance and the complementarity of combinatorial engineering compared to rational engineering, for which one would never choose these amino acids as targets to increase enzyme activity.

3.5. Biochemical characterisation of improved variants

The genes encoding 002-G1 and 001-D2 variants were subcloned into pGEX-6P3 to increase the expression level and to allow direct affinity purification using GST tag, as described previously [2]. GST fusion proteins were purified from 500 ml cultures, yielding 30 mg pure protein for each variant. The specific activity (determined in the presence of 146 mM sucrose and 0.1 g/l glycogen) obtained from cultures of the two variants is increased five times compared to the wild-type, but the specific activity of the purified enzymes is 3.8 and 4.2 times higher than the wild-type, for clones 001-D2 and 002-G1, respectively (Table 3). These data indicate that the expression level of the two variants is slightly higher than for the wild-type. Indeed, the screening protocol does not make it possible to distinguish enzyme efficiency and gene expression level.

Although the screening was performed in the presence of the activator glycogen, the kinetic parameters of the action on sucrose reveal that the increased efficiency was partly conserved for activity on sucrose alone (Table 4). The very sensi-

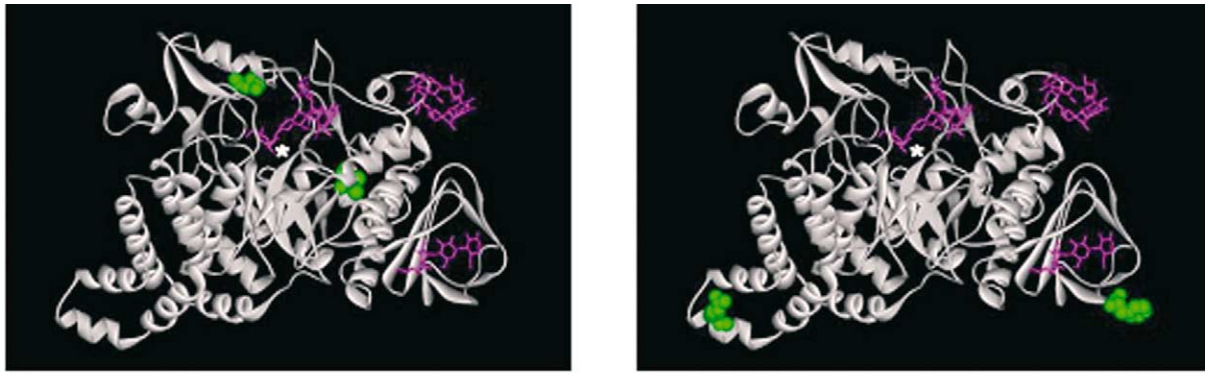

Fig. 4. Position of the mutated residues (green) in clones 001-D2 (A) and 002-G1 (B). The active site is indicated by the white asterisk; oligosaccharides at specific binding sites are represented in purple.

tive method used for the detection of glucose and fructose makes it possible for the first time to differentiate the hydrolysis and polymerisation reactions catalysed by amylosucrase. The glucose formation reflects the hydrolysing activity, since it can only be formed when water is used as acceptor. The fructose formation reflects the total consumption of sucrose, and thus the total activity (sucrose is not used as acceptor by the enzyme and use of fructose as acceptor to produce sucrose isomers occurs only at high fructose concentrations and not in the initial stages of the reaction). The fructose formation minus glucose formation then reflects the polymerisation activity. Although the kinetic profile of amylosucrase action on sucrose does not present a classical Michaelian behaviour, it is possible to model this profile by two different Michaelis–Menten equations (Fig. 5) [3]. These reveal a high affinity and low V_{max} at low sucrose concentrations (V_{max1} and K_{m1}) and low affinity and high(er) V_{max} at high sucrose concentrations (V_{max2} and K_{m2}). In the kinetic parameters shown in Table 4,

Table 3
Purification of (variant) enzymes

	Culture			Purified enzyme			
	Vol (ml)	Act.[a] (U/ml)	Spec. Act. (U/mg)	Spec. Act. (U/mg)	Protein (mg)	Purification factor	Yield (%)
001-D2	500	0.60	2.4	6.4	30	2.6	64
002-G1	500	0.58	2.5	7.2	30	2.9	73
Wild-type	5000	0.22	0.5	1.7	570	3.5	90

[a]Act.: activity under standard conditions (146 mM sucrose+0.1 g/l glycogen).

Table 4
Kinetics of the action on sucrose of (variant) enzymes

	K_{m1} (mM)	V_{max1} (U/mg)	k_{cat1}/K_{m1}	K_{m2} (mM)	V_{max2} (U/mg)	k_{cat2}/K_{m2}
Total activity						
Wild-type	4.0	0.62	0.19	71	1.2	0.020
001-D2	2.5	0.96	0.46	61	1.7	0.033
002-G1	2.9	0.84	0.34	55	1.3	0.029
Hydrolysis						
Wild-type	2.5	0.29	0.14	29	0.43	0.018
001-D2	2.2	0.51	0.28	22	0.65	0.035
002-G1	2.7	0.42	0.19	27	0.53	0.023
Polymerisation						
Wild-type	8.1	0.36	0.05	112	0.75	0.008
001-D2	2.9	0.44	0.19	117	1.11	0.011
002-G1	3.2	0.43	0.16	79	0.81	0.012

$k_{cat} = 1.2 \times V_{max}$

Table 5
λ_{max} and absorption (in parentheses) at λ_{max} after iodine staining of products formed from different concentrations of sucrose by (variant) enzymes

mM Suc	5	10	20	50	100	200	500
Wild-type	nd	nd	560 (0.06)	575 (0.35)	570 (0.22)	555 (0.14)	550 (0.17)
001-D2	nd	580 (0.13)	600 (0.33)	595 (0.19)	580 (0.10)	540 (0.10)	555 (0.18)
002-G1	nd	575 (0.16)	585 (0.31)	590 (0.29)	590 (0.22)	575 (0.16)	560 (0.19)

nd, no polymer formation detected.

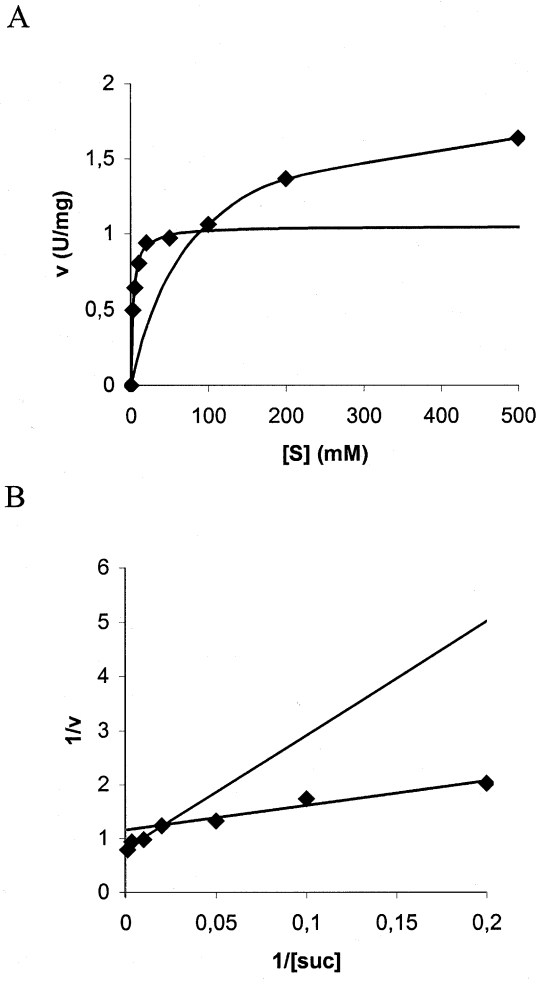

Fig. 5. The typical v versus [S] plot (A) and Lineweaver–Burk plot (B) of the action of amylosucrase on sucrose shown here for variant 001-D2.

the most significant changes caused by the mutations occur in the catalytic efficiency (k_{cat}/K_m) at both low and high concentrations of sucrose when looking at total activity. However, at higher sucrose concentrations both mutants show a more significant increase in hydrolysing activity, whereas at lower sucrose concentrations the polymerisation efficiency is more increased. This is reflected in the polymer formation by the mutants, which is similar to that by the wild-type at high sucrose concentrations, but significantly improved at low sucrose concentrations (Table 5). Whereas the wild-type does not produce any polymer from 10 mM sucrose, both variants do. Furthermore, from 20 and 50 mM sucrose the variants appear to produce longer amylose chains than the wild-type, judged by the increase in λ_{max}. The iodine staining depends on the structure of the glucan, with a shift from red to blue with decrease in branching. Since amylosucrase only makes linear chains, a higher λ_{max} suggests the presence of longer amylose chains.

4. Conclusions

Combinatorial engineering of amylosucrase has been started to optimise its catalytic properties. A zero background expression cloning strategy has been developed, which allows us to make huge variant libraries. To explore the large variant space created by optimised error-prone PCR and gene shuffling, simple and rapid selection and screening methods have been developed and miniaturised for automation. A convenient method for detailed kinetic analysis of amylosucrase action on sucrose has been adapted for use in microtitre plates to allow rapid and extensive comparison between wild-type and variant enzymes.

Finally, a first small-scale screening using sucrose and glycogen as substrate resulted in the selection of two improved variants with significantly increased activity on sucrose alone. Furthermore, the two variants are more activated by the presence of an acceptor such as glycogen than the wild-type, as shown by the four-fold increased activity for both variants in these conditions. This suggests an interesting potential of these variants to be used for the modification of glucans.

Sequence analysis revealed that they contained mutations in regions that would not have been selected for directed mutagenesis, emphasising the strength of this strategy, which will aid our search for amylosucrase and other glucansucrase variants with improved catalytic efficiency, increased thermostability, and decreased side product formation.

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