High level expression of a recombinant amylosucrase gene and selected properties of the enzyme

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Abstract

Two high-level heterologous expression systems for amylosucrase genes have been constructed. One depends on sigma-70 bacterial RNA polymerase, the other on phage T7 RNA polymerase. Translational fusions were formed between slightly truncated versions of the gene from *Neisseria polysaccharea* and sequences of expression vectors pQE-81L or pET33b(+), respectively. These constructs were introduced into different *E. coli* strains. The resulting recombinants yielded up to 170 mg of dissolved enzyme per litre of culture at a moderate cell density of five OD₆₀₀. To our knowledge, this is the highest yield per cell described so far for amylosucrases. The recombinant enzymes could rapidly be purified through the use of histidine tags in the N-terminally attached sequences. These segments did not alter catalytic properties and therefore need not be removed for most applications. Investigations with glucose and malto-oligosaccharides of different lengths identified rate-limiting steps in the elongation (acceptor reaction) and truncation (donor reaction) of these substrates. The elongation of maltotriose and its reversal, the truncation of maltotetraose, were found to be particularly slow reactions. Potential reasons are discussed, based on the crystal structure of the enzyme. It is furthermore shown that amylosucrase is able to synthesize mixed disaccharides. All of the glucose epimers mannose, allose and galactose served as acceptors, yielding between one and three main products. We also demonstrate that, as an alternative to the use of purified AS, cells of the constructed recombinant strains can be used to carry out glucosylations of acceptors.

Keywords

Amylosucrase, High level expression, Glucosylation, Acceptor, Rate-limiting step, Whole cells.

Introduction

An amylosucrase (AS) was first isolated from bacteria of the genus *Neisseria*. These enzymes are glucosyltransferases (EC 2.4.1.4) that use sucrose (Suc) to catalyze the formation of amylose-like glucans containing α -1,4-linkages (Hehre et al. 1949; MacKenzie et al. 1978; Okada and Hehre 1974; Tao et al. 1988). This is done by transferring the D-glucose residue (Glc) of sucrose onto a Glc or malto-oligosaccharide (MO) molecule (Albenne et al. 2004). These elongation products then serve as acceptors for the next transfer and so forth. D-fructose (Fru), which is liberated by these reactions, is also used as acceptor. It differs from the acceptor Glc in two respects. Firstly, the regiospecificity of the reaction is relaxed, so that, besides the re-formation of Suc, two Suc isomers, turanose and trehalulose, are formed (Okada and Hehre 1974; Potocki de Montalk et al. 2000b). Secondly, these disaccharides do not appear to be further elongated.

ASs consist of a single polypeptide chain of about 640 amino acids (AAs) (Ha et al. 2009; Pizzut-Serin al. 2005; Potocki de Montalk et al. 1999; Zhu et al. 2003). They belong to family 13 of glycoside hydrolases and, together with family 70, which comprises the other known glucansucrases, and family 77, form the clan or superfamily H (Henrissat 1991; Henrissat and Davies 1997). Probably all of its members contain a $(\beta/\alpha)_8$ barrel as common fold. Three-dimensional structures of the AS from *N. polysaccharea* have been determined (Jensen et al. 2004; Mirza et al. 2001; Skov et al. 2001; Skov et al. 2002). Catalysis by AS proceeds via a two-step mechanism. Firstly, a reaction between the enzyme and the glycosyl donor forms a covalent AS-glucosyl intermediate. This subsequently reacts with an acceptor. These two nucleophilic substitutions result in a retention of the α -configuration at the anomeric carbon (Jensen et al. 2004).

Biotechnological applications as well as high-throughput screens in micro formats (Schneider et al. 2009) require the availability of efficient producer strains. Convincing demonstrations of high level expression, such as SDS-PAGE analyses of cell extracts, are either lacking or show rather low AS yields (Büttcher et al. 1997; Potocki de Montalk et al. 1999; Ha et al. 2009). Moreover, the only system described for affinity chromatography of the enzyme uses the rather bulky GST tag (Potocki de Montalk et al. 1999). We therefore set out to construct high-level recombinant expression systems that use the small His tag for purification. The isolated AS was used to verify a potential influence of the tag on the enzymatic activity and its stimulation.

We furthermore characterized selected properties of the enzyme or the newly constructed strains, respectively. One was the identification of rate-limiting steps in both, the elongation and its reversal, the truncation of MOs. Previous experiments (Albenne et al. 2004; Schneider et al., 2009) had shown that the synthesis of MOs does not proceed at an even, chain-length-independent rate. We now investigated these reactions in greater detail by systematically challenging the enzyme with MOs of different degrees of polymerization. A largely unknown property of the enzyme is its acceptor spectrum. As a first step into this field, we investigated if epimers of Glc can be transformed into mixed disaccharides. Finally, we examined if the need to purify the enzyme could be entirely circumvented by employing the newly constructed recombinant strains directly, i. e., in the form of whole cells, for the glucosylation of acceptors.

Materials and methods

Chemicals including oligonucleotides

Chemicals were of the highest purity available. Biochemical grade glycogen with a molecular mass range between 0.27 and 3.5×10^6 was obtained from Merck (Darmstadt, Germany). Primers were purchased from von MWG Biotech (Ebersberg, Germany) or Invitrogen (Karlsruhe, Germany).

Bacterial strains

Neisseria polysaccharea ATCC 43768 (Riou et al. 1983) was the source of the AS gene. *E. coli* XL10-Gold (Stratagene, Amsterdam, Netherlands) was used as primary host in the construction of pJEA1000 (see below). *E. coli* BL21*[DE3] (Invitrogen, Karlsruhe, Germany) was used as host for pJEA1000 for expression of its AS gene. *E. coli* DH5 α (Grant et al. 1990) and ElectroTen-Blue (Stratagene, Amsterdam, Netherlands) were used as hosts for pJQA1000 (see below).

Culture conditions

N. polysaccharea was grown at 37 °C in brain heart infusion medium, consisting of 37 g/l brain heart infusion (Difco Laboratories, Augsburg, Germany), and under air containing 5 % carbon dioxide. *E. coli* strains were routinely cultivated at 37°C in LB medium (Sambrook and Russel 2001), supplemented with the appropriate antibiotics. For AS production, transcription was induced by addition of IPTG to 0.4 mM when an OD₆₀₀ of 0.7-0.9 was reached. Cells were harvested after an additional incubation for 4 h at 30°C.

Isolation of the AS gene and plasmid constructions

DNA manipulations were carried out by standard techniques as described by Sambrook and Russell (2001). The AS gene was amplified from genomic DNA of *N. polysaccharea* with *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany), using the forward primer GTCGGTTTGAATTTCACAGTACCTC and the reverse primer CATTCGGGAAGCTTGCGTCAGGC, which introduced *Eco*RI and *Hind*III sites (underlined, with the respective point mutations in bold) and a small 5'-terminal truncation. The PCR product was cleaved with the two endonucleases. The *Sgr*AI site of the vector pET33b(+) (Novagen, WI, USA) was removed by cleavage with this enzyme, fill-in of the ss ends, and recircularization. The resulting plasmid was digested with *Eco*RI and *Hind*III and dephosphorylated. All products were purified with QIAquick columns (Qiagen, Hilden, Germany) and ligated to yield pJEA1000. The integrity of the AS gene was confirmed by DNA sequencing. It was excised from pJEA1000 with *Bam*HI and *Hind*III. After isolation by agarose gel electrophoresis, it was ligated with pQE-81L (Qiagen, Hilden, Germany) that had been cleaved with the same endonucleases, dephosphorylated and purified with a QIAquick column. This yielded pJQA1000.

Determination of AS activity

AS activity was determined enzymatically with a reagents kit from r-biopharm (Darmstadt, Germany). The protocol is based on the procedure of Mayer (1987), modified to separately quantitate Fru and Glc liberation. Substrate hydrolysis was monitored by measurement of Glc formation; transglucosylation was determined from the difference between Fru and Glc

liberation. In the standard procedure, aliquots of cell extracts or purified enzymes were incubated at 30°C for 15 min in phosphate-buffered saline, consisting of 8 mM Na₂HPO₄, 140 mM NaCl, 2.7 mM KCl and 1.5 mM KH₂PO₄, pH 7.3, supplemented with 150 mM of Suc. When primers were present, their concentrations were 0.1 g/l for glycogen and 150 mM for α-methyl-D-glucoside (α-Me-Glc). Further details of the assay have previously been described (Swistowska et al. 2007). One unit of activity was defined as the amount of enzyme forming 1 μmol of Fru per min under assay conditions. For determination of intra- and extracellular activity of resting cell preparations, cells were pelleted by centrifuging at 13000 g for 3 min. For extraction of AS, they were treated with B-PER (Thermo Fisher Scientific, St. Leon-Rot, Germany) according to the protocol of the supplier. After another centrifugation as above, the resulting supernatant and the first supernatant, obtained by pelleting of the intact cells, were used for activity determination in the presence of glycogen as described above.

Incubations of various substrates with purified AS

Basic reaction conditions were as described for the activity assay. Incubations of Glc or MOs were carried out for up to 20 h with 30 or 150 mM of substrate and 0.8 or 1.6 U AS per ml. When Suc was additionally present, its concentration was 150 mM. Glucosylation tests of monosaccharides were carried out for 24 h with 25 mM Suc, 500 mM monosaccharide, and 1.6 U AS per ml. Reactions were analyzed by thin-layer chromatography (TLC) and/or high performance liquid chromatography (HPLC) as given below.

Glucosylations with resting cells

Recombinant strains BL21*[DE3](pJEA1000) or ElectroTen-Blue(pJQA1000) were grown at 37 °C to an OD₆₀₀ of about 0.8. After addition of IPTG to 0.4 mM, the incubation was continued O/N at 30°C. Cells were harvested, washed and finally suspended in 50 mM sodium phosphate, pH 7.0. Transformations were carried out in the same buffer at 30°C and a cell density of 1 OD₆₀₀. Substrate concentrations were 150 mM Suc and 15 mM acceptor.

Thin-layer chromatography (TLC)

Plates used were Silica gel 60 F₂₅₄ with concentration zone (Merck, Darmstadt, Germany). Mobile phases were A, ethyl acetate/2-propanol/water (50/40/10, v/v/v) or B, acetonitrile/ethyl acetate/1-propanol/water (85/20/50/60, v/v/v/v) (Robyt and Mukerjea 1994). One or two ascents were carried out. Detection of carbohydrates was done as previously described (Mukerjea et al. 1996).

High performance liquid chromatography (HPLC)

Column chromatography was carried out with a Shimadzu LC10AD instrument (Shimadzu Corp., Kyoto, Japan) equipped with refractive index and diode array detectors. Flow rates were 1 ml/min. Separations of α-(para-nitrophenyl)-D-glucoside (α-pNP-Glc) and its products were done isocratically with 10 % of aqueous methanol on an SC Lichrosphere 100 RP8 5 μm column (length: 125 mm, ID: 4.6 mm) (Bischoff, Leonberg, Germany). Separations of MOs and their products were done isocratically with 60 % of aqueous acetonitrile on a ProntoSIL 120-3-Amino 3 μm column (length: 125 mm, ID: 4.6 mm) (Bischoff, Leonberg, Germany). Amounts were quantitated from HPLC peak areas.

Staining of His-tagged proteins

201 Visualization of His-tagged proteins was done with the InVision His-tag In-gel Stain
202 (Invitrogen). The microwave procedure was carried out according to the supplier's
203 instructions, with minor modifications. Excitation was done with a UV-Transilluminator at
204 254 nm.

205
206 Other methods

207
208 Enzyme purification (Schneider et al. 2009), DNA sequencing (Bartels et al. 1999),
209 determination of protein concentration, using bovine serum albumin as standard (Bradford
210 1976) and protein gel electrophoresis and quantitation (Swistowska et al. 2007) have
211 previously been described.

Results

Improvement of recombinant AS yields

The AS gene was obtained by PCR amplification from genomic DNA of *Neisseria polysaccharea* ATCC 43768 (Riou et al. 1983). In order to achieve high-level expression, two different *E. coli* plasmids were constructed, one where transcription relied on the host's sigma-70 RNA polymerase, and another where transcription depended on the phage T7 RNA polymerase. The first was obtained by insertion of the AS gene into vector pQE-81L, resulting in plasmid pJQA1000. The second was constructed by cloning the gene in vector pET33b(+), a *SgrA*⁺ derivative of pET33b(+), resulting in plasmid pJEA1000. Details of these constructions are given in "Materials and methods". The integrity of the gene was verified by sequencing. In both constructs transcription is controlled by the *lac* repressor, which is encoded by each of the plasmids. Both expression vectors also code for N-terminal His-tags. Therefore the insertions of the AS gene were designed to generate translational fusions with these sequences. As a consequence, both tagged enzymes differ from the WT in a few N-terminal AAs that were replaced by vector-encoded AAs. These differences, as deduced from the DNA sequences, are shown in Table 1.

E. coli strains DH5 α (Grant et al. 1990) and ElectroTen-Blue (Stratagene) were used as hosts for pJQA1000-directed AS biosynthesis. The enzyme concentration obtained was similar in both hosts; the most abundant cellular protein present in dissolved form was AS (Fig. 1). From one litre of culture of an OD₆₀₀ of five, 50 to 60 mg of AS were obtained.

E. coli BL21*[DE3] was used as host for pJEA1000-directed AS biosynthesis. The expression level of the gene was even higher in this system (Fig. 1), which is probably due to the high amount of transcripts synthesized by the T7 RNA polymerase in conjunction with the RNaseE-deficient phenotype of the host. The target enzyme constituted about 30 % of the total dissolved cellular protein (see below). A time course experiment showed that the growth rate was not significantly changed by induction of AS gene transcription and that the highest concentration of the enzyme was reached four to five hours after the addition of IPTG and remained constant for at least a further 15 h (not shown). Typically, about 170 mg of AS were obtained from one litre of culture of an OD₆₀₀ of five.

A His-tag-specific stain after SDS-PAGE confirmed the integrity of the purification handle (not shown). As expected from the crystal structure of the enzyme (Skov et al. 2001), the N-terminal tags were sterically accessible for binding to a Ni²⁺ matrix. A one-step purification yielded an approximately 90 % pure enzyme. The balance of a typical AS isolation from 40 ml of a culture of strain BL21*[DE3](pJEA1000) is shown in Table 2. The low purification factor reflects the high percentage of target protein in the crude extract.

Catalytic activity of His-tagged AS and its stimulation

In numerous experiments carried out we never observed differences in behaviour between the two His-tagged enzymes, encoded by either pJQA1000 or pJEA1000. Thus properties given below refer to both recombinant enzymes. Specific total, transfer and hydrolytic activities of the His-tagged enzymes were determined with Suc alone and with glycogen or α -Me-Glc as additional acceptor substrates (Table 3). The value of the specific total activity determined in the presence of 0.1 g/l of glycogen, which represents the standard assay conditions, was somewhat lower than the value determined during the purification (Table 2). An intermediate value (9565 U/g) has been reported for AS after removal of a GST-tag (Potocki de Montalk et al. 1999). In view of inevitably occurring variations due to loss of activity during purification and storage, these differences cannot be regarded as significant. Slightly lower values for the

three specific activities were determined when glycogen was replaced by 150 mM of the acceptor α -Me-Glc (Table 3).

The known activating effect of glycogen on AS activity was determined as a function of its concentration (not shown). When glycogen concentrations were repeatedly increased by a factor of 10, activity reached a plateau value above 1 g/l of glycogen. The maximal enhancement measured was about three orders of magnitude. The curve shape and the maximal activity reached (about 72000 U/g) were very similar to the data reported for the de-tagged enzyme mentioned above (Potocki de Montalk et al. 2000a). A general difference was that in our experiments a given activity value was reached at a lower glycogen concentration, which is presumably due to the use of a glycogen preparation with higher acceptor efficiency.

Our results did not indicate any significant differences between the catalytic behaviour of the de-tagged enzyme (Potocki de Montalk et al. 2000a) and of the His-tagged AS described here.

Alternative acceptors

The formation of turanose and trehalulose by AS (Okada and Hehre 1974; Potocki de Montalk et al. 2000b) indicates that the acceptor specificity of the enzyme is to a certain extent relaxed. Therefore the Glc epimers mannose, allose, and galactose, which differ from Glc in the configuration at C2, C3 or C4, respectively, were examined as acceptors. A 20-fold excess of these monosaccharides over Suc was used to keep the background originating from only Suc-dependent products low. After complete consumption of Suc, formation of elongation products was detected with all of the three substrates. One product was formed from mannose, three from allose, and two from galactose (Fig. 2). TLC mobility of the products suggests that they contain one, two or three Glc moieties, respectively. While, due to the specificity of the enzyme, it is almost certain that the Glc residues have been attached by α linkages, it is presently unclear whether bonds have been formed with C4 of the acceptors. More material is required for characterizations by NMR.

Elongation and truncation of MOs

Malto-oligosaccharides do not only serve as acceptors for further Glc additions, they also glucosylate the enzyme in a reversal of the elongation reaction (Albenne et al. 2002). In order to elucidate the influence of substrate length on both reactions, chain elongation and truncation, AS was separately challenged with Glc and MO di- to heptamers in the absence and/or presence of Suc. Aliquots taken at various time points were analyzed by TLC and HPLC.

Fig. 3 shows a typical result and illustrates the main findings. The broad distributions of elongation and truncation products that were formed indicate that both reactions proceeded in a largely non-processive manner. Non-processivity in chain elongation has also been concluded by Albenne et al. (2004) from product formation in the presence of Suc as sole substrate. As can be seen in Fig.4, the product distributions in the truncation and elongation reactions showed significant differences in concentrations between certain MOs. Particularly slow donor reactions were observed with MO2 and MO4, as evident from the accumulations of these oligomers. Otherwise, these rates generally increased with the length of the donor. Similarly, the rates of elongations typically increased with the length of the acceptor. Glc (not shown in Fig.4) also fits into this scheme as it was a much less efficient acceptor than MO2. MO3, however, represents an exception to the rule. It was a significantly slower acceptor than MO2, as indicated by its exceptionally high concentrations (Fig. 3). The relatively slow elongation of MO3 also manifests itself in a broader distribution of elongation products than observed with MO4. In summary, the slowest of these reactions were the truncation of MO2

together with its reversal, the elongation of Glc, and the truncation of MO4 together with its reversal, the elongation of MO3. AS structural features that may be responsible for these characteristics are proposed in the Discussion section.

Transglucosylations by AS with whole recombinant cells

Resting cells of *E. coli* ElectroTen-Blue(pJQA1000), BL21*[DE3](pJEA1000) and the untransformed hosts were prepared and incubated with Suc as donor and acceptors such as α -Me-Glc or α -pNP-Glc. Aliquots of the supernatants were analyzed. Whereas no substrate conversions by the host strains were detectable (not shown), the presence of the plasmids resulted in conversion of the acceptors into a range of elongation products. They were the same as formed by the purified enzyme. An example with α -pNP-Glc as acceptor is shown in Fig. 4. Similar to the results obtained with the oligosaccharides that do not contain an aglycon (Fig. 3), an accumulation of the product containing three Glc units was observed.

Determination of intra- and extracellular AS activity of resting cell preparations showed differences between the two strains. While 6.6 % of the total AS activity (360 U/l) was found in the supernatant of BL21*[DE3](pJEA1000), these values were 45 % of 300 U/l for ElectroTen-Blue(pJQA1000). Rather different data are found in the literature with respect to an intra- or extracellular localisation of AS produced by *E. coli* cells (Büttcher et al. 1997; Potocki de Montalk et al. 1999). Our results suggest that these may be due to the use of different strains. No attempt was made in the current work to determine to what extent the extracellular activity was due to secretion of the enzyme or to cell lysis. We note, however, that strain BL21*[DE3](pJEA1000), which showed an activity of only 24 U/l in the resting cell supernatant, formed more glucosylation products from α -Me-Glc or α -pNP-Glc than strain ElectroTen-Blue(pJQA1000), which yielded 130 U/l in the supernatant. This indicates that the intracellular activity of the former strain was mainly responsible for product formation.

Discussion

Efficient heterologous expression of genes is still largely a question of trial and error. As frequently the initiation of translation is a limiting factor, the generation of translational fusions with efficiently translated 5'-terminal sequences is a promising strategy. Making use of previous experience with the high-level expression of the glucansucrase type *gtfR* gene from *Streptococcus oralis* (Swistowska et al. 2008), we pursued such an approach and successfully constructed two efficient AS gene expression plasmids for bacterial hosts. AS yields obtained were substantially higher than described in previous publications that reported comparable data (Büttcher et al. 1997; Potocki de Montalk et al. 1999; Champion et al. 2009; Ha et al. 2009). After reaching maximal values, the high AS levels were stable over a wide time window. The attached purification tags were functional. A removal of the tags and of additional vector-derived segments prior to use of the enzymes was not required, as there was no significant alteration of catalytical properties by these sequences.

The glycosylation experiments with whole recombinant cells show that there was no detectable interfering activity of the host. Although, depending on the host strain used, different fractions of AS activity were found in the supernatant, our results suggest that intracellular activity played a major role for glucosylation of α -Me-Glc, α -pNP-Glc and their products. From the practical point of view, we can assert, on the basis of several transformation experiments that have successfully been carried out, that incubations of acceptors with resting cells proved to be a convenient way to achieve efficient glucosylations. Products could be recovered in relatively pure form from the supernatants, particularly as incubations were carried out in a simple, defined medium. These observations suggest that in many cases glucosylations may advantageously be carried out with easily preparable cells, obviating the need to isolate the enzyme.

Monitoring of product formation from substrates of increasing chain length permitted the identification of rate-limiting steps in elongation or truncation reactions. Two previous publications have reported similar investigations. Albenne et al. (2002) examined the disproportionation of MO2 to MO7 by AS and found a general increase of rates with substrate length and a particularly large enhancement between MO4 and MO5. These experiments, however, were unable to distinguish which of the disproportionation reactions, elongation or truncation, were rate-limiting. Albenne et al. (2004) characterized the kinetics of product formation with Suc as the only substrate. They observed lag phases for the formation of MO3, MO4, and particularly longer oligomers. Our present experiments, carried out with defined starting concentrations of Glc and MOs and in the absence as well as in the presence of Suc, showed that particularly slow elongation reactions are the conversions of the mono- into the dimer and of the tri- into the tetramer. They also demonstrated that, in contrast to the results reported by Albenne et al. (2004), there are no lag phases for the elongations of MO4 and longer oligomers. Our experiments further showed that the slowest truncation reactions are the reversals of the above-mentioned steps, i. e., the conversions of the di- into the monomer and of the tetra- into the trimer. Crystal structures have been determined for the unliganded AS as well as, among others, for the AS-Glc intermediate and the enzyme with an MO7 molecule bound at the active site (Jensen et al. 2004; Skov et al. 2001; Skov et al. 2002). The access tunnel from the AS surface to the active site comprises five subsites that are able to accommodate Glc residues. Each of these becomes occupied as the MO chain grows (Skov et al. 2002). The donor Suc occupies subsite -1 with its Glc moiety and subsite +1 with its furanose form Fru moiety. For elongation, the terminal Glc moiety of an acceptor has to be accommodated by the latter subsite. According to the crystal structures with Suc (PDB code 1JGI) or MO7 (PDB code 1MW0) as ligands, the subsite possesses the same structure when harbouring a Fru or a Glc residue. The formation of the Suc isomers turanose and trehalulose by AS shows that Fru can also be bound in subsite +1 in the pyranose form and in different

orientations. This relaxed specificity suggests that the positioning of Glc at this site will not be optimal, providing a rationale for the relatively slow truncation of MO2 and for the slow elongation of Glc. The additional subsites presumably have evolved for optimal interactions with the growing glucan chain and therefore are likely to provide additional interactions that positively affect the binding of longer donor and acceptor molecules. This agrees with the observed general trend of increases in elongation and truncation rates with chain length. However, there is an exception to this rule. MO3, acting as acceptor, or MO4, acting as donor, are rather poor substrates. Both reactions require occupation of subsite +3. A comparison of the positions of all MO7-lining AAs (structure 1MW0) with their positions in three structures without a bound MO molecule (1G5A, 1JGI, 1S46) showed essentially identical positions of these AAs with two major exceptions, Arg226 and Thr398. In the latter three structures, both AAs occupy positions that would sterically interfere with Glc residues in subsites +2 or +3, respectively. This suggests that the binding of MOs to these sites has to displace these AA residues. In the case of Arg226, rotation of the side chain suffices to avoid clashes with the MO molecule. In contrast, the main chain of Thr398 and of adjacent residues must be displaced to accommodate a Glc residue in subsite +3. Our experimental data suggest an interference at subsite +3 rather than +2. We therefore propose that clashes between the Glc unit in subsite +3 and Thr398, as depicted in Fig. 5a, are responsible for the observed reductions of reaction rates. Our data further showed that contacts in subsite +4 overcompensate the negative interaction in subsite +3. Phe399 and Asp401 are likely candidates for strong interactions with the Glc moiety in subsite +4, forming hydrogen bonds with O6 and O2, respectively (Fig. 5b). It has been proposed that Arg415 strengthens the MO binding at subsite +4 by hydrophobic interaction (Albenne et al. 2004). The importance of this residue may also lie in the formation of hydrogen bridges with the aforementioned Phe399 and Asp401, thereby ensuring their correct positioning.

Very little is known about the acceptor range of AS. Previous experiments showed that Glc, MOs and Fru can be glucosylated by AS (Okada and Hehre 1974; Potocki de Montalk et al. 2000b). Glc and MOs may carry an aglycon at their reducing end (Albenne et al. 2002). Our current work demonstrates that Glc transfers to other sugars are also catalyzed by the enzyme. The monosaccharides mannose, allose and galactose served as acceptors. Moreover, initial experiments suggest that also different disaccharides can be elongated by AS. The formation of these mixed saccharides is further experimental evidence for a relaxed specificity for substrate binding in subsite +1, as mentioned above.

To summarize, two highly efficient expression systems for AS genes are available. The resulting gene products carry short functional tags for facile purification which do not interfere with catalytic activity and thus must not be removed for most applications.

The newly constructed strains can also be employed in the form of whole cells to carry out acceptor glucosylations.

In the early stages of chain elongation by AS, two steps are particularly slow, glucosylation of the mono- and of the trimer. Similarly, the reversals of both reactions proceed at exceptionally low rates, demonstrating that MO2 and MO4 are poor Glc donors.

AS is able to transfer Glc to carbohydrates other than Glc, MOs and Fru. The finding that mannose, allose and galactose, and probably also some of their acceptor products as well as other disaccharides, serve as acceptors may open up a novel route for the preparation of mixed oligosaccharides.

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Figure Legends

Fig. 1 Expression of the genes encoding His-tagged amylosucrases. Strains were grown as described in "Materials and methods". Total protein in raw extracts was analyzed by 12.5 % SDS-PAGE and staining with Coomassie Blue. Lane M, marker proteins; lane 1, BL21*[DE3](pET33b+); lane 2, BL21*[DE3](pJEA1000); lane 3, DH5 α (pQE-81L); lane 4, DH5 α (pJQA1000); lane 5, ElectroTen-Blue(pQE-81L); lane 6, ElectroTen-Blue(pJQA1000). Amounts loaded correspond to 5 μ l of cell suspensions of 30 OD₆₀₀. Molecular masses of marker proteins are given at the left margin. The position of the AS band is marked by an arrow.

Fig. 2 Analysis of acceptor function of Glc epimers. Incubation mixtures were separated by TLC with solvent A. Substrates used (mannose, Man; allose, All; galactose, Gal) are given above the lanes. + and - designate with or without incubation. Marker lanes are labeled M. Positions of acceptor products are highlighted by dots.

Fig. 3 Intermediates of MO truncation and elongation by AS. Samples were taken after 20 h of incubation with 0.8 U/ml of AS and were analyzed by TLC with solvent B. Lanes M1, marker containing Glc, Fru, Suc, MO2 and MO3; lanes M2, marker containing MO di- to heptamers; lanes MO2 to MO7, incubations of AS with the indicated MO (150 mM) only; lanes MO2* to MO7*, incubations of AS with the indicated MO plus Suc (150 mM). Each educt is marked by a dot left of the respective lane.

Fig. 4 Acceptor glucosylation by whole cells. *E. coli* ElectroTen-Blue(pJQA1000) was incubated with 15 mM α -pNP-Glc as acceptor and 150 mM Suc as donor. Sampling times are indicated below the lanes. Aliquots were analyzed by TLC with solvent B. For further details see Materials and methods.

Fig. 5 Structural interpretation of the kinetics of MO elongation and truncation. a. Stereo view of steric interference of a Glc residue in subsite +3. Subsite -1 is at the bottom of the figure. Glc moieties in subsites -1 to +4 of a MO7 molecule are shown in CPK representation, with carbons in green for subsite +3 and in yellow for other subsites. AAs within 4 Å around the Glc residues are shown as sticks, except for Thr398, shown in CPK representation. The figure is based on PDB structures 1G5A (AAs) and 1MW0 (MO7). b. Stereo view of hydrogen bonding pattern between AS and MO7 bound at the active site. Only AAs probably forming hydrogen bonds (indicated as dashed green lines) with the substrate are shown. Carbons of MO7 are in yellow. Note the additional hydrogen bonds of the Glc moiety in subsite +4 with Phe399 and Asp401. The figure is based on PDB structure 1MW0. Both figures were drawn with PyMOL (DeLano, 2007).

Table 1 Differences in the N-terminal sequences of amylosucrases. Protein sequences are deduced from the DNA sequences. Vector-encoded AAs are underlined

Enzyme	N-terminal sequence
AS-WT	MLTPTQQVGLILQ...
His-tagged AS (pJQA1000)	<u>MRGSHHHHHHTDPNSQ</u> ...
His-tagged AS (pJEA1000)	<u>MGSSHHHHHHSSGLVPRGSRRASVHMASMTGGQQMGRDPNSQ</u> ...

Table 2 Balance of one-step purification of AS from strain BL21*[DE3](pJEA1000).^a

Fraction	Protein mass [mg]	AS activity ^b [U]	AS specific activity ^b [U/g]	Purification factor
Cell extract	22.1	69.1	3120	1.00
After buffer exchange	6.1	75.0	12300	3.94

^a From 50 ml of a culture of 5 OD₆₀₀.

^b Determined under standard conditions.

Table 3 Specific activity of His-tagged AS in the absence and presence of different acceptor molecules

Acceptor		Specific activity [U/g]					
Type	Concentration	Total		Transfer		Hydrolysis	
		mean	SD	mean	SD	mean	SD
-	-	421	9	14.4	4.8	407	10
α -Me-Glc	150 mM	7489	74	7109	70	380	5
Glycogen	0.1 g/l	8890	135	8460	127	421	8

Fig. 1

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Fig. 2

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Fig. 3

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Fig. 4

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