Amylosucrase from *Neisseria polysaccharea*: novel catalytic properties

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Abstract Amylosucrase is a glucosyltransferase that synthesises an insoluble α-glucan from sucrose. The catalytic properties of the highly purified amylosucrase from *Neisseria polysaccharea* were characterised. Contrary to previously published results, it was demonstrated that in the presence of sucrose alone, several reactions are catalysed, in addition to polymer synthesis: sucrose hydrolysis, maltose and maltotriose synthesis by successive transfers of the glucosyl moiety of sucrose onto the released glucose, and finally turanose and trehalulose synthesis – these two sucrose isomers being obtained by glucosyl transfer onto fructose. The effect of initial sucrose concentration on initial activity demonstrated a non-Michaelian profile never previously described.

Key words: Amylosucrase; Sucrose; α-1,4-Glucan; Oligosaccharide; Non-Michaelian profile

1. Introduction

Amylosucrase is a glucosyltransferase (EC 2.4.1.4), which synthesises an insoluble α-1,4-linked glucan polymer from sucrose, and releases fructose. Unlike most amylopolysaccharide synthases [1], this enzyme does not require any α-D-glucosyl-nucleotide-diphosphate, such as ADP- or UDP-glucose [2]. Indeed, amylosucrase only uses the energy produced by the splitting of the osidic linkage of sucrose to synthesise other osidic linkages.

Amylosucrase was first discovered in cultures of *Neisseria perflava* [3]. In 1974, *Neisseria polysaccharea* was isolated from the throat of healthy children in Europe and Africa [4]. This strain was shown to possess an extracellular amylosucrase, that synthesises an amylose-like polymer from sucrose [5]. Further, the gene encoding amylosucrase from *N. polysaccharea* was cloned and expressed in *Escherichia coli* [5–7]. The study of its sequence revealed that this enzyme is constituted by a $(\beta/\alpha)_8$-barrel [7], and belongs to family 13 of the glycosylhydrolases [8–12]. Particularly, the amylosucrase sequence contains the corresponding characteristic invariable catalytic triad, and several conserved amino acids involved in the activity of amylolytic enzymes [7,13,14]. The catalytic mechanism of amylosucrase may therefore resemble that of α-amylases, especially for the formation of the intermediate glucosyl–enzyme complex. Moreover, amylosucrase shares common features with all other sequence-known glucansucrases from *Leuconostoc* sp. and *Streptococcus* sp. [7,15,16]. Nevertheless, the structure prediction revealed that it is the only glucan-sucrase whose $(\beta/\alpha)_8$-barrel is not circularly permuted [7,17]. Consequently, amylosucrase constitutes an essential model for the investigation of the relations between structure and specificity of glucansucrases, as it is situated at the interface between amylolytic enzymes and other glucansucrases.

Few studies have dealt with the kinetic behaviour of amylosucrase in the presence of sucrose as sole substrate. The first study, carried out with the constitutive enzyme obtained from crude extracts of *N. perflava* [18], showed that a lag phase occurred before the initiation of the glucose polymerisation reaction. The fructose production catalysed by the recombinant enzyme of *N. polysaccharea*, obtained from crude extracts of *E. coli*, did not reveal the presence of a proper lag phase, but the fructose production was found to be significantly enhanced after 5 h of reaction [6]. Nevertheless, the purified enzyme, free from potential contaminating activities, had never been previously studied. Moreover, until now, all kinetic studies had been carried out using reducing sugar assays [6,18] to quantify fructose release versus time. Indeed, authors considered that the stoichiometry of the reaction was conserved between the sucrose consumed, and the amylopolysaccharide and fructose produced. Thus, production of glucose and/or oligosaccharides had never been described before the present study.

In this paper, we present a thorough investigation of the catalytic properties of amylosucrase, purified to homogeneity, and thus free from any contaminating activity. For the first time, all the products synthesised from sucrose by amylosucrase are characterised, revealing novel catalytic properties of the enzyme. Finally, the atypical kinetic behaviour of amylosucrase with respect to its natural substrate is described.

2. Materials and methods

2.1. Bacterial strains

The gene encoding amylosucrase came from *N. polysaccharea* ATCC 43768 chromosomal DNA. Cloning of the amylosucrase gene was carried out as previously described [7]. *E. coli* strain BL21 was used as the host of pGEX-6-P-3 for fusion protein expression.

2.2. Enzyme extraction methods

Cultured recombinant bacteria were centrifuged (8000×*g*, 10 min, 4°C). In order to extract the intracellular enzyme, the bacterial pellet was resuspended and concentrated at OD_{600nm} 80, in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). After sonication, 1% (v/v) Triton X-100 was added to the extract. After 30 min at 4°C and centrifugation (10 000×*g*, 10 min, 4°C), the supernatant was stored at 4°C.

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2.3. Purification of amylosucrase

Affinity chromatography between glutathione *S*-transferase/amylosucrase (GST-AS) fusion protein and the glutathione-Sepharose-4-B support was performed to purify the amylosucrase as previously described [7]. After purification, the fusion protein was cleaved by the PreScission Protease (Amersham Pharmacia Biotech). The released GST and the protease were finally separated from amylosucrase by binding onto the glutathione-Sepharose-4-B support, pure amylosucrase being eluted. This purification process allowed single-band electrophoresis gels to be obtained, in native and denaturing conditions, for which proteins were stained with 0.5% (w/v) $AgNO_3$ [7]. Part of the same batch of purified amylosucrase was used for crystallisation [19]. Amylosucrase molecular mass is 70 kDa. The purified enzyme retained 95% of its activity after 7 months storage at −80°C, and 53% at 4°C. Amylosucrase activity was optimal between pH 6.8 and 7.3. The optimum temperature was 30°C, and the enzyme retained 92% of its activity after 5 h at 30°C. Consequently, all the kinetic data were recorded in less than 5 h. During this interval, initial rate measurements were not affected by amylosucrase stability. Indeed, the half-life of the enzyme was 21 h at 30°C, and it was inactive after 48 h. However, the recombinant amylosucrase from *N. polysaccharea* is much more stable than the native and partially purified enzyme from *N. perflava* strain 19–34, for which a half-life of 15 to 20 h at 4°C was previously reported [18].

2.4. Amylosucrase assay

The amylosucrase assay, using 0.04 mg/ml purified amylosucrase, was carried out at 30°C in 50 mM Tris–HCl, pH 7.0, supplemented with 1 to 600 mM sucrose, and with 0 to 20 mM additional fructose to study the effect of fructose. One unit of amylosucrase corresponds to the amount of enzyme that catalyses the consumption of one μmol of sucrose per min in the assay conditions. The hydrolysis rate corresponds to the amount of free glucose released versus time. The determination of the initial rates led to a maximal experimental error of 7%. The polymerisation rate has been determined by difference between the total sucrose consumption rate and the hydrolysis reaction rate (maximal error 13%).

2.5. Oligosaccharide production

Oligosaccharide synthesis was performed using 100 mM sucrose and 0.2 mg/ml pure amylosucrase. After 48 h at 30°C (99% sucrose consumption), the medium was centrifuged (20 000 × *g*, 40 min), to eliminate insoluble polysaccharide, and freeze-dried. Sugars were then resuspended in water to 450 g/l and the pH adjusted to 4.5 with 1 M HCl. Invertase (I-4504, Sigma Chemical Co.) was then added (426 U/ml) to the medium to hydrolyse any residual sucrose. The reaction was performed at 55°C for 24 h.

2.6. Oligosaccharide purification

Separation was performed on a preparative 250 × 20 mm octadecyl reverse-phase chromatography column (C18 column) (Bischoff Chromatography). Nanopure water was used at 5 ml/min. Multiple 500 μl injections of the medium (450 g/l soluble sugars) were carried out, and 1 ml fractions were collected and submitted to analytical HPLC to verify their purity (maximal error 5%).

2.7. Oligosaccharide hydrolysis with amyloglucosidase and maltase

Enzymatic oligosaccharide hydrolysis was used to characterise the structure of the oligosaccharides synthesised from 106 mM sucrose, after 48 h of reaction (reaction supernatant S). Using amyloglucosidase (15 U/ml, Ref. No. 1202332, Boehringer Mannheim) or maltase (7 U/ml, G-4634, Sigma Chemicals), the enzymatic hydrolysis was carried out at 25°C for 15 h, in the reaction supernatant S, diluted 1.3 times, and containing 50 mM Tris–HCl buffer, the pH being adjusted with HCl to 4.7 and 6.0, respectively.

2.8. Carbohydrate analysis

Sucrose, glucose and fructose concentrations were measured by ion-exchange chromatography at 25°C with an Aminex HP87H column (Biorad Chemical Division, Richmond, CA, USA), the eluant being 8.5 mM H_2SO_4, at 0.5 ml/min. Oligosaccharide separation was performed with a 250 × 4 mm octadecyl reverse-phase chromatography column (Bischoff Chromatography), eluted with water at 0.5 ml/min. The experimental error for HPLC analyses did not exceed 1 mM. Product characterisation was performed in a 4 × 250 mm Dionex Carbo-pack PA1 column. Mobile phase NaOH (150 mM) was used at 1 ml/min flow rate, with a sodium acetate gradient (6 to 180 mM in 20 min). Detection was performed using a Dionex ED40 module with a gold working electrode and a Ag/AgCl pH reference. The maximal experimental error for Dionex analyses was 1 mM.

2.9. Polymer production and characterisation

α-Glucan synthesis was carried out at 30°C, in 50 mM Tris–HCl buffer, pH 7.0, containing 100 mM sucrose and 200 mg/l purified amylosucrase. After 48 h reaction, the polymer was precipitated by adding 10 vol of absolute ethanol at 4°C. The polymer (10 g/l) was solubilised in 1 M KOH at 4°C during 15 h. The solution was then neutralised by addition of 9 vol 0.1 M HCl. Polymer debranching was carried out with isoamylase from Hayashibara (10 U/mg polymer), in sodium citrate buffer (25 mM, pH 3.8) and 5 mM $CaCl_2$, during 40 h at 45°C. The molecular mass of the polymer was determined by size-exclusion chromatography using the HPSEC system (three Shodex OHpak KB-800 series columns, Showa Denko K.K., Tokyo, Japan), as previously described [7]. Dual detection of solutes was carried out with a light-scattering detector and a differential refractive index detector [7].

2.10. NMR spectroscopy

^{13}C spectra were recorded on a Brucker AC300 spectrophotometer (operating frequency, 75 468 MHz). The polysaccharide sample was dissolved to 30 g/l in dimethylsulfoxide.

3. Results and discussion

3.1. Characterisation of reaction products

In order to characterise all the reaction products, the reaction was first carried out in the presence of 106 mM sucrose for 48 h. A white insoluble polymer, which was subsequently characterised, was synthesised. The medium was centrifuged to separate the polymer and the reaction medium supernatant (sample S) was analysed by Dionex HPLC. The chromatogram which was obtained (Fig. 1) revealed the presence of seven different products, named P1 to P7. The retention times of products P1, P2 and P4 correspond, respectively, to glucose (resulting from sucrose hydrolysis), fructose and residual sucrose. These results were confirmed by HPLC using Aminex and C18 columns (data not shown).

3.1.1. Characterisation of compounds P3, P5, P6 and P7. When analysed by Dionex HPLC (Fig. 1B) and by reverse-phase chromatography on a C18 column, products P6 and P7 presented the same retention times as maltose and maltotriose, respectively. Moreover, reaction medium supernatant S was submitted to maltase action, which preferentially hydrolyses α-1,4-linked glucooligosaccharides, but can also hydrolyse α-1,2, α-1,3 and α-1,6 linkages. Only compounds P6 and P7 were hydrolysed. The same result was obtained after the action of amyloglucosidase, which hydrolyses only α-1,4 and α-1,6 linkages between glucosyl residues. Finally, compound P7 was purified by HPLC on a preparative C18 column, and analysed by ^{13}C NMR. The ^{13}C NMR spectrum of P7 corresponded exactly to the spectrum of maltotriose described by Heyraud et al. [20]. As a result, it can be concluded that amylosucrase catalysed the synthesis of maltose and maltotriose by successive transfers onto glucose residues released by hydrolysis of sucrose.

Products P3 and P5 were not separated on C18 column HPLC. Both presented the retention time characteristic of oligosaccharides of degree of polymerisation (DP) 2. In addition, they were totally resistant to the action of maltase and amyloglucosidase. On the Dionex chromatogram (Fig. 1B), products P3 and P5 were compared to the five sucrose iso-

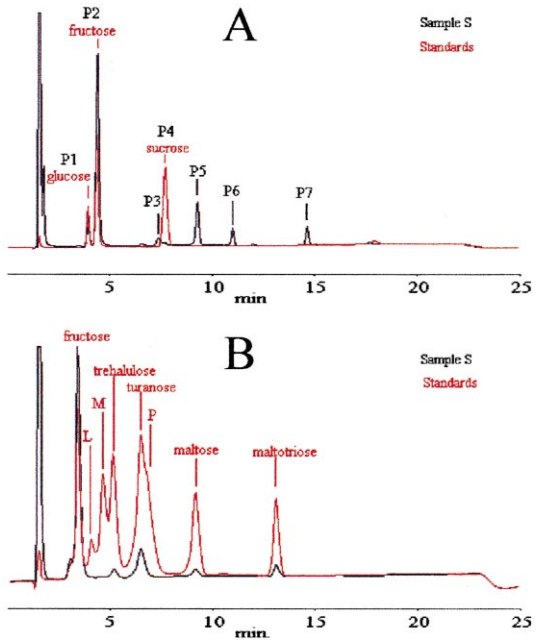

Fig. 1. Dionex HPLC profile of the reaction medium supernatant, after 48 h reaction in the presence of 106 mM sucrose (sample S). Standards are composed of glucose, fructose and sucrose (panel A), and of leucrose (L) (5-*O*-α-D-glucopyranosyl-D-fructose), maltulose (M) (4-*O*-α-D-glucopyranosyl-D-fructose), trehalulose (1-*O*-α-D-glucopyranosyl-D-fructose), turanose (3-*O*-α-D-glucopyranosyl-D-fructose), palatinose (P) (6-*O*-α-D-glucopyranosyl-D-fructose), maltose and maltotriose (panel B).

mers. The retention time of P3 corresponded to that of trehalulose. The standard mixture used in Fig. 1B contained turanose and palatinose, which were not separated by this technique. These two sucrose isomers were tested separately, showing that product P5 was probably turanose. Trehalulose was synthesised only in very small proportions, but large quantities of turanose were produced by amylosucrase at the end of the reaction (Table 1). Other glucansucrases catalyse the synthesis of sucrose isomers. Particularly, dextransucrase from *Leuconostoc mesenteroides* synthesises large quantities of leucrose in the presence of sucrose and fructose [21], and smaller quantities of isomaltulose and trehalulose [22]. Production of isomaltulose and trehalulose from sucrose was observed in the case of dextransucrase from *Streptococcus bovis* [23].

Turanose is a rare sucrose isomer, which is acariogenic, like other sucrose isomers [24]. In addition, it can be used for medical diagnosis of Pompe's disease as an inhibitor of the deficient 'acid α-glucosidase' of kidneys and leukocytes [25]. Moreover, until now, its chemical synthesis was too tedious [26] to envisage its application on an industrial scale like trehalulose, leucrose and isomaltulose. Consequently, the use of amylosucrase may constitute a novel and very promising way to synthesise turanose.

3.1.2. Polymer characterisation. The polymer synthesised from sucrose (100 mM) by electrophoretically pure amylosucrase was analysed by ^{13}C NMR. Chemical shifts were characteristic of exclusively α-1,4-linked glucosyl units, as referenced by Bock et al. [27]. Indeed, no signal corresponding to an α-1,6-linked glucosyl residue was observed. Moreover, the polymer precipitated with ethanol was submitted to isoamylase action. No debranching products were detected by Dionex HPLC control, confirming that the α-glucan is totally exempt from α-1,6 linkages. Consequently, the structure of the α-glucan synthesised from sucrose by the recombinant amylosucrase from *N. polysaccharea* strongly differs from that produced by *N. polysaccharea* itself, which was described to be an amylopectin-like polymer [28]. *N. polysaccharea* probably possesses both an amylosucrase and a branching enzyme, as it was recently suggested for *Neisseriae* species [29].

Size-exclusion chromatography coupled to light-scattering resulted in a calculated average molecular mass of the α-glucan of 8941 g/mol, corresponding to a DP 55, and a polydispersity of 1.1. This result is in agreement with the maximal absorption wavelength obtained at 575 nm for the glucan–iodine complex, the value obtained for a DP 60 α-1,4-glucan being 576 nm. Nevertheless, the calculated polymer size differed significantly from the results obtained by Büttcher et al. [5], who concluded that two populations of α-glucans were synthesised from 146 mM sucrose by crude enzymatic extracts from recombinant *E. coli* cells. The average DPs of these populations were found to be 3000 and 3800, respectively.

3.2. Kinetic studies

The release of maltooligosaccharides and sucrose isomers was followed versus time by HPLC using Aminex and C18 columns. At the beginning of the reaction, during the determination of initial activity, fructose release was found to be equimolar with sucrose consumption. During this initial step, only two reactions occurred: polymerisation and sucrose hydrolysis. Indeed, maltooligosaccharides and sucrose isomers appeared in the medium after a longer time than that used for the determination of the initial activity. Consequently, acceptor reactions did not interfere with kinetic measurements. Fructose and glucose appeared immediately after the addition of amylosucrase to the medium, and the sucrose consumption rate did not increase with reaction time. These results are not in agreement with previously published data, obtained with crude enzyme extracts of amylosucrase [6,18]. The lag phase observed by the authors could thus be due to the presence of interfering enzyme activities.

In addition, no maltooligosaccharide of DP higher than 3

Table 1
Reaction yields after 48 h reaction in the presence of 106 and 10 mM sucrose

Product	Initial sucrose: 106 mM		Initial sucrose: 10 mM	
	Consumed sucrose: 105 mM		Consumed sucrose: 10 mM	
	mM	Yield (%)	mM	Yield (%)
Glucose	4.4	4	3.0	30
Maltose	3.6	7	1.5	29
Maltotriose	4.6	13	0.6	18
Trehalulose	2.2	2	0	0
Turanose	17.3	17	1.1	11
Glucan		57		12
Total		100		100

Yields correspond to the ratio between the total glucosyl units incorporated into the product and the total glucosyl units transferred from sucrose. The synthesised glucan being insoluble, the corresponding yield is obtained from the difference between the total consumed glucosyl units and those incorporated into glucose and oligosaccharides.

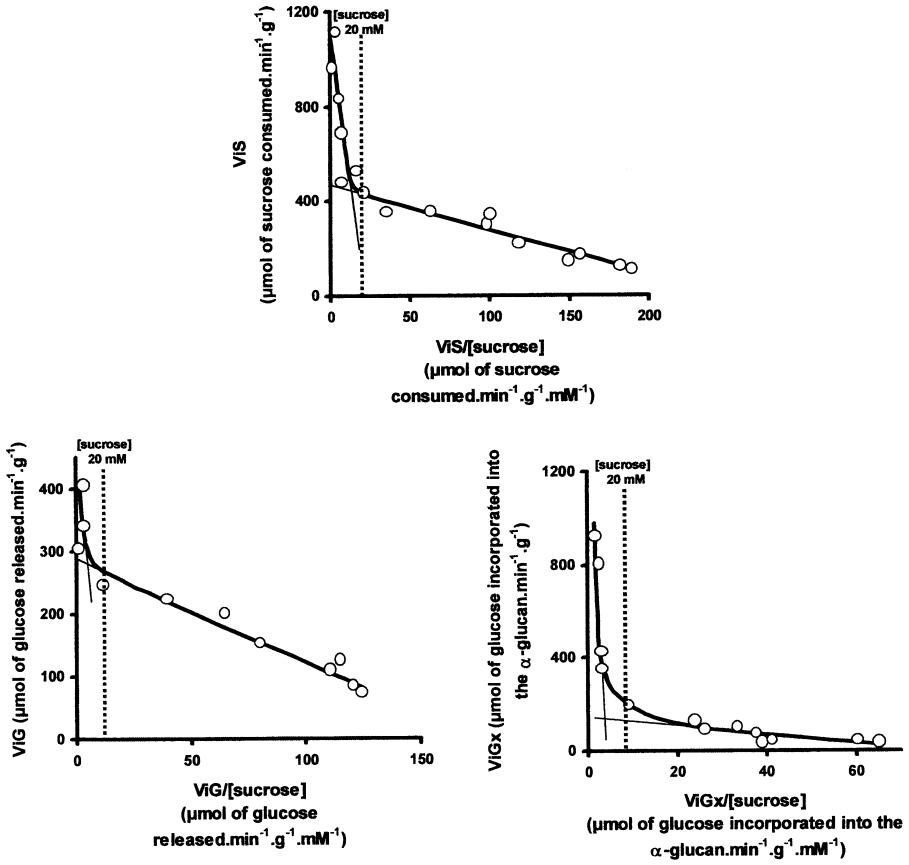

Fig. 2. Eadie–Hofstee representations for the initial rate of sucrose consumption (ViS), the initial rate of glucose release (ViG), and the initial rate of glucose incorporation into the α-glucan (ViGx).

was detected at any reaction time in the medium, indicating that the polymerisation reaction follows a processive mechanism.

3.2.1. Effect of sucrose concentration. The Eadie–Hofstee representation (Fig. 2) clearly demonstrates that amylosucrase does not present a classic Michaelis-Menten behaviour for sucrose consumption. Indeed, the slope of the linear curve obtained for initial sucrose concentrations lower than 20 mM was shallower than that obtained for higher initial sucrose concentrations. Similar profiles were obtained for the initial rates of glucose release and polymer synthesis. Nevertheless, it is possible to model sucrose consumption rate versus sucrose concentration by two different Michaelis-Menten equations. For initial sucrose concentrations lower than 20 mM, the apparent Michaelis constant (K_m) was 1.9 mM, and the apparent catalytic constant (k_{cat}) was 33 min^{-1}. The values of K_m and k_{cat} obtained for initial sucrose concentrations higher than 20 mM were 50.2 mM and 77 min^{-1}, respectively. The apparent kinetic constants for sucrose hydrolysis and polymerisation are presented in Table 2.

The effect of initial sucrose concentration on the distribution between hydrolysis and polymerisation reactions during initial activity determination was studied. Initial hydrolysis rate represents 73% of the initial sucrose consumption rate in the presence of 6 mM sucrose, 63% for 20 mM sucrose, and only 28% for 295 mM sucrose. These data reveal that amylosucrase favours hydrolysis at low sucrose concentrations, and polymerisation at high sucrose concentrations. These observations are in agreement with the results presented in Table 1. Indeed, the proportions of glucose and then of

Table 2
Apparent kinetic values for sucrose consumption, sucrose hydrolysis and polymerisation initial rates

Initial [sucrose]	Apparent kinetic constants	ViS	ViG	ViGx
< 20 mM	K_m	1.9 mM	1.7 mM	1.9 mM
	V_{max}	470 µmol of sucrose consumed min^{-1} g^{-1}	288 µmol of glucose released min^{-1} g^{-1}	147 µmol of glucose incorporated into the α-glucan min^{-1} g^{-1}
	K_{cat}	33 min^{-1}	20 min^{-1}	10 min^{-1}
> 20 mM	K_m	50.2 mM	38.7 mM	387 mM
	V_{max}	1100 µmol of sucrose consumed min^{-1} g^{-1}	472 µmol of glucose released min^{-1} g^{-1}	1620 µmol of glucose incorporated into the α-glucan min^{-1} g^{-1}
	K_{cat}	77 min^{-1}	33 min^{-1}	113 min^{-1}

oligosaccharides (30 and 58% of the consumed sucrose, respectively) were higher for 10 mM initial sucrose, than for 106 mM initial sucrose (4 and 39% of the consumed sucrose).

This atypical kinetic behaviour of amylosucrase could be related to the presence of a second sucrose binding site. This non-catalytic site could be occupied by sucrose only at high substrate concentrations and could induce conformational changes, leading to an increase of the initial reaction rate and to a modification of the balance between hydrolysis and polymerisation.

3.2.2. Effect of fructose concentration. The Lineweaver-Burk representations (for initial sucrose concentrations between 20 and 100 mM) (data not shown) are characteristic of competitive inhibition by fructose, with an apparent inhibition constant (K_i) of 14 mM. This is in agreement with what was previously described by MacKenzie et al. [30,31], who observed competitive inhibition by fructose with the constitutive enzymes from eight *Neisseriae* species (K_i between 15 and 50 mM, depending on the species).

4. Conclusion

In the presence of sucrose as sole substrate, pure amylosucrase presents complex and never previously described catalytic properties. They result in the synthesis of a large diversity of products, synthesised by several different reactions, and in a non-Michaelian kinetic behaviour for sucrose. This atypical catalytic mechanism deserves further investigation, particularly at the molecular level. Indeed, the identification of the enzyme–substrate and enzyme–product interaction sites will allow, in the long term, the selection of unique reaction pathways through a site-directed mutagenesis approach.

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