Action pattern and subsite mapping of *Bacillus licheniformis* α-amylase (BLA) with modified maltooligosaccharide substrates

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Abstract This study represents the first characterisation of the substrate-binding site of *Bacillus licheniformis* α-amylase (BLA). It describes the first subsite map, namely, number of subsites, apparent subsite energies and the dual product specificity of BLA. The product pattern and cleavage frequencies were determined by high-performance liquid chromatography, utilising a homologous series of chromophore-substituted maltooligosaccharides of degree of polymerisation 4–10 as model substrates. The binding region of BLA is composed of five glycone, three aglycone-binding sites and a 'barrier' subsite. Comparison of the binding energies of subsites, which were calculated with a computer program, shows that BLA has similarity to the closely related *Bacillus amyloliquefaciens* α-amylase.

Key words: α-Amylase; 2-Chloro-4-nitrophenyl; Benzylidene; Maltooligosaccharide; Bond-cleavage frequency; Binding energy

1. Introduction

The α-amylases (EC 3.2.1.1) comprise a group of enzymes that catalyse the hydrolysis of the α-1,4 glycoside linkages in starch and related carbohydrates with retention of the α-anomeric configuration in the products. They are found in family 13 of the classification of glycosyl-hydrolases according to Henrissat [1]. The structure of family 13 enzymes consists of a characteristic $(\beta/\alpha)_8$-barrel catalytic core domain with a varying number of extra domains [2,3].

α-Amylases can be found in microorganisms, plants and animals where they play a dominant role in carbohydrate metabolism. These enzymes and related amylolytic enzymes are widely used in biotechnology for starch degradation [4] and in synthetic chemistry for the formation of oligosaccharides by transglycosylation [5–7]. Furthermore, these enzymes are used as targets for drug design in attempts to treat diabetes, obesity, hyperlipemia and caries [8].

Bacillus licheniformis is a mesophilic organism but produces a highly termostable α-amylase (BLA) [9]. Therefore, it is of great significance in present day biotechnology. It has several industrial applications. It is used in alcohol, sugar and brewing industries for the initial hydrolysis of starch [4].

At the three-dimensional level, the crystal structure of BLA has been described [10,11], together with the structure of calcium-depleted BLA [12]. In spite of the extensive studies concerning the structure and thermal properties of BLA [13,14] and the numerous reports in the literature referring to the molecular mechanism of irreversible thermoinactivation [15–17], little attention has been paid to its enzymological characterisation. The action pattern of BLA was studied by paper chromatography using starch and linear maltooligosaccharides [18,19]; however, the cleavage frequency of the glycosidic bonds has not been quantified.

Present studies were aimed at determining the action pattern and product specificity of BLA by utilising as model substrates 2-chloro-4-nitrophenyl (CNP) β-glycosides of maltooligosaccharides of degree of polymerisation (DP) 4–10 and two 4-nitrophenyl (NP) derivatives modified at the non-reducing end with a 4,6-*O*-benzylidene (Bnl) group. These results are discussed on the basis of the popular subsite model, introduced by Phillips et al. [20] in the study of lysozyme and Schechter and Berger [21] in their work on proteinase papain.

A computer program was also developed in our laboratory according to the computer model of Allen and Thoma [22,23] and was applied to the subsite mapping of BLA.

2. Materials and methods

2.1. Substrates

The homologous maltooligomer substrate series 1, 2 (Fig. 1) (DP 4–8) were synthesised from cyclodextrins [24]. The longer chain length of CNP-maltooligosides in the range of DP 8–10 was prepared by a chemoenzymatic procedure using rabbit skeletal muscle glycogen phosphorylase b [25].

2.2. Enzyme

α-Amylase (EC 3.2.1.1) from *B. licheniformis* type XII-A (Sigma) gave a single band on SDS–PAGE and possessed no α- and β-glycosidase activity.

2.3. Hydrolysis of the maltooligosides

Incubations in 25 mM glycerophosphate buffer (pH 7.0) containing 5 mM $CaCl_2$ and 50 mM NaCl were carried out at 50°C for 2, 4, 6 and 8 min. The reactions were initiated by the addition of 10 μM of enzyme to solutions containing 1.7 mM of substrate. Samples were taken at the indicated time intervals and the reaction was stopped by the injection of the samples into the chromatographic column. In these studies we have taken care to exclude the secondary attacks

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Abbreviations: BAA, *Bacillus amyloliquefaciens* α-amylase; BCF, bond-cleavage frequency; BLA, *Bacillus licheniformis* α-amylase; Bnl, 4,6-*O*-benzylidene; CNP, 2-chloro-4-nitrophenyl; DP, degree of polymerisation; NP, 4-nitrophenyl

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on the substrates. The product ratios were always obtained from the early stages of hydrolysis (conversion $< 10\%$), before any secondary attack could be detected.

2.4. Chromatographic analysis

A Hewlett-Packard 1090 Series II liquid chromatograph equipped with a diode array detector, automatic sampler, and ChemStation was used for high-performance liquid chromatography (HPLC). Samples were separated on a Supelco NH_2 5 μm column (20×0.46 cm) with gradient elution of MeCN-water flowing at a rate of 1 ml/min at 40°C. Effluent was monitored for the NP/CNP group at 302 nm and the products of the hydrolysis were identified by using relevant standards. The quality of the acetonitrile was gradient grade. Purified water was obtained from a laboratory purification system equipped with both ion-exchange and carbon filters (Millipore, Bedford, MA, USA).

2.5. Calculation of subsite map

Subsite mapping is simplified for exo-enzymes because there is only one productive binding mode for each substrate. However, endo-enzymes form more productive binding modes resulting in a complex product pattern. The relative rate of formation of each product is called bond-cleavage frequency (BCF), which gives information about subsite-binding energy. By using BCFs for a series of oligomeric substrates, it is possible to calculate the subsite-binding energy for every subsite on the enzyme-binding region, with the exception of the two subsites adjacent to the catalytic site which are occupied by every productive complex.

A computer program was also evaluated for subsite map calculation on the basis of synopsis of Allen and Thoma [22,23]. SUMA (subsite mapping for α-amylases) runs in Windows environment and uses the experimentally determined BCFs for determination of the number of subsites, the position of the catalytic site and for calculation of subsite affinities. The apparent free energy values were optimised by minimisation of the differences of the measured and the primary calculated BCF data.

3. Results and discussion

Detailed knowledge about subsite architecture of BLA is scarce. No report on kinetics and mode of action of this industrially important enzyme can be found in the literature. Therefore, the use of modified, low-molecular weight substrates should be an effective way to elucidate the number of subsites in the active site area of BLA, which has not been reported yet.

We found that maltohexaose and higher maltooligosaccharides were good hydrolytic substrates, but maltopentaose and maltotetraose were considered poor substrates. Kinetic parameters were determined on linear maltooligosaccharides of DP 5–8 (K_M values of 2.5, 3.3, 4.0, 5.0 mM and V_{max} values of 0.0045, 0.14, 0.33, 0.5 mM/min were obtained for maltopentaose, maltohexaose, maltoheptaose and maltooctaose, respectively).

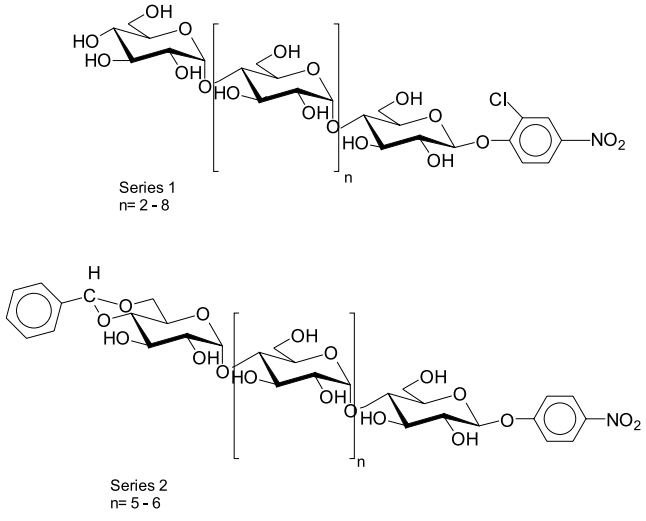

Fig. 1. Structure of modified oligosaccharides. Series 1: CNP β-maltooligosaccharides; series 2: Bnl-NP β-maltooligosaccharides.

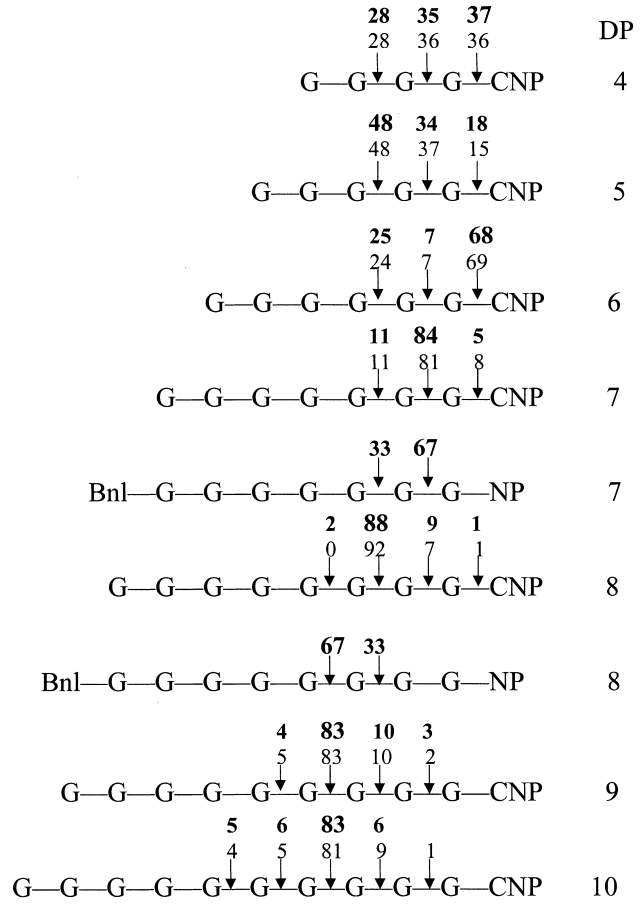

Fig. 2. Action pattern of BLA with modified maltooligosaccharides at 50°C. G, glucosyl residues; NP, 4-nitrophenyl groups; CNP, 2-chloro-4-nitrophenyl groups (NP and CNP are connected to the reducing end in β-form); Bnl groups (connected to the non-reducing end as Bnl acetals); DP, degree of polymerisation. Numbers above the arrows represent percentages of cleavage frequencies of glycosidic bonds. Bold numbers are experimental data, the bigger ones indicate the most favourable positions of cleavages. Normal numbers are related to the calculated data based on the optimised subsite map.

3.1. Action pattern and BCFs of BLA on CNP-maltooligosaccharides

In the course of our studies of convenient substrates for α-amylases, CNP and Bnl-modified NP (Bnl-NP) β-maltooligosaccharides DP 4–8 were synthesised and used for the study of the active centre of PPA [26] and HSA [27].

CNP-maltooligosaccharides of longer chain length, in the range of DP 8–11, were obtained by a transglycosylation reaction catalysed by rabbit skeletal muscle phosphorylase b using α-D-glucopyranosyl-phosphate as a donor [25]. The primer in the enzymatic reaction was CNP β-maltoheptaoside (G_7-CNP), synthesised from β-cyclodextrin using a convenient chemical method [24]. These maltooligomer substrates are in-

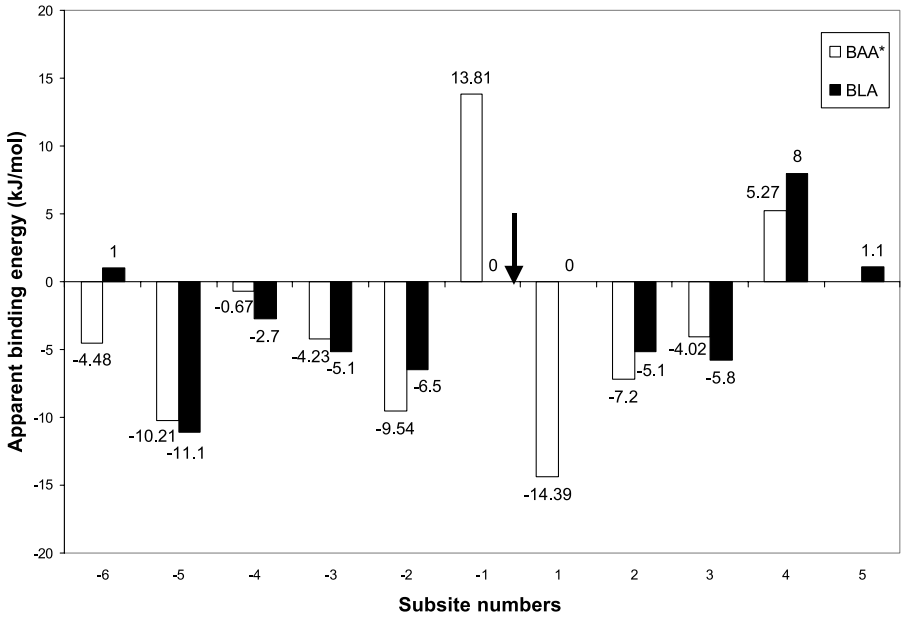

Fig. 3. Subsite maps for BAA* and BLA (*according to Allen and Thoma [23]). The open bars depict the subsite map for BAA and the solid bars are related to BLA. The arrow indicates the location of hydrolysis. The reducing end of maltooligomers situated at the right hand of the subsite map. Negative energy values indicate bindings between the enzyme and aligned glucopyranosyl residues, while positive values indicate repulsion.

dispensable tools in the investigation of binding sites and the actions of α-amylases having a longer binding area than that of human α-amylases.

The series of CNP-maltooligosaccharides DP 4–10 and two Bnl-protected derivatives of NP β-maltooligosaccharides DP 7 and 8 (Fig. 1) were used in the BLA reaction to determine unambiguously the exact glycosidic linkage being cleaved, as well as the cleavage frequency which, as an indicator of the binding mode of the corresponding substrate, was tested by HPLC. The concentration of glycosidic fragments produced by BLA reaction showed linearity with the reaction time, and the distribution of products was calculated for a given substrate. Reproducible values were obtained at four incubation times; and the mean values are given in Fig. 2.

BLA exhibits a unique pattern of action on CNP-maltooligosaccharides by cleaving maltopentaose units as main products; 68%, 84% and 88% from the non-reducing end of CNP-G_6, CNP-G_7 and CNP-G_8, respectively, and leaving CNP-glycosides.

As the chain length increases, the maximum frequency of attack shifts toward the reducing end of the chain and CNP-G_3 becomes the major product; 88%, 83% and 83% from CNP-G_8, CNP-G_9 and CNP-G_{10}, respectively. This favourable release of CNP-G_3 was also observed for the pentamer glycoside (CNP-G_5). In this case the substrate was too short to occupy all the subsites and three glycosyl residues from the reducing end were bound in subsites (+1, +2, +3) and led to the formation of CNP-G_3 in 48%. The release of CNP-G_3 is much more favourable than the release of CNP-G_2 and CNP-G_1, 34% and 18%, respectively.

Our results strongly suggest the presence of at least eight subsites in BLA, five glycone sites (−5, −4, −3, −2, −1) and three aglycone sites (+1, +2, +3) and the catalytic site is located between subsites (−1 and +1). The subsites are labelled from the catalytic site, with negative numbers for subsites to the left (non-reducing end side) and positive numbers to the right (reducing end side) according to the recently proposed nomenclature of Davies et al. [28].

For all the substrates the chromogenic aglycones (CNP/NP), which were in β-glycosidic linkages, could interact with subsites (+2 and +3), but less favourably than a glucose residue. In the ideal arrangement subsite (+3) was filled by a glycopyranosyl unit and the aglycone sites (−5, −4, −3, −2, −1) were also occupied by glucose residues which resulted in an interesting dual product specificity for the dominant formation of CNP-G_3 and maltopentaose.

3.2. Subsite mapping of BLA

For subsite mapping we applied the procedure of Allen and Thoma [22,23]. BCFs were evaluated for a chain length of 4–10 of CNP β-maltooligosides and these quantitative data were used to calculate the subsite map for BLA. Primarily calculated binding energies were optimised to give an optimum fit of BCFs to the experimentally measured values. Fig. 3 shows the subsite maps and the apparent binding energies of subsites for BLA and *Bacillus amyloliquefaciens* α-amylase (BAA) [23].

The subsites (−6), (+5) of BLA have +1.0 and +1.1 kJ/mol free energy, respectively. We can suggest that subsites (−6) and (+5) are not real subsites for BLA, but (−6) is a real one for BAA. Both enzymes have another subsite which interacts unfavourably with the glucose unit. Subsite (+4) has positive free energy of binding and will be referred to as 'barrier subsite'. This barrier subsite resulted in the dual product specificity of BLA and BAA. Lack of the 'barrier subsite' leads to a more equal distribution between the potential products of the longer substrates as we found in HSA [27]. Results confirm that the eight subsites originally assumed from our experimental data are correct and BCFs are predicted correctly within experimental error (Fig. 2).

3.3. Patterns of action and BCFs of BLA on the Bnl-blocked NP-maltooligosaccharides (Bnl-NP)

The product pattern of Bnl oligomers (Fig. 2) was interesting and different from that of the unmodified substrates. Only one prominent glycosidic fragment was released from the unprotected glycosides. The main hydrolysis products of G_7-CNP and G_8-CNP were 84% G_2-CNP and 88% G_3-CNP, respectively, while those from BnlG_7-NP and BnlG_8-NP were both only 67%. These suggest an unfavourable interaction between the Bnl group and subsite (−6) and confirm the presence of the five glycone-binding subsites.

It was envisaged that the Bnl group would not mimic a glucopyranosyl unit, but we found that it can occupy subsite (−5). Hydrolysis of BnlG_7-NP and BnlG_8-NP also resulted in 33% G_3-NP and 33% G_2-NP, respectively, compared to 11% G_3-CNP and 9% G_2-CNP when G_7-CNP and G_8-CNP, respectively, were hydrolysed. It can be assumed that both ends of the active site (−7 and +4 sites) contain hydrophobic regions which are advantageous to the apolar residues (Bnl, CNP/NP). The dual product specificity of BLA could be observed on the benzylidene-modified substrates, as well, which resulted in the dominant formation of BnlG_5 or G_3-NP.

α-Amylase from *B. licheniformis* was an attractive model enzyme for investigating the active centre. The subsite map of BLA on modified homologous oligosaccharides provided further insight into the structure and function of BLA.

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