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Action pattern of α-amylases on modified maltooligosaccharides

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2-Chloro-4-nitrophenyl- (CNP) and 4,6-O-benzylidene-modified 4-nitrophenyl- (Bnl-NP) β-maltooligosaccharides (DP 4-8) were synthesised from cyclodextrins using a chemical procedure. For the preparation of CNP-maltooligosides of longer chain length a new chemoenzymatic procedure was developed using rabbit skeletal muscle glycogen phosphorylase b. These substrates were used for further studies of the action pattern of porcine pancreatic α-amylase (PPA), human salivary α-amylase (HSA) and *Bacillus licheniformis* α-amylase (BLA). The hydrolysis products and the remaining substrates were separated and quantified by HPLC. Our results suggest at least six subsites in the binding region of HSA; four glycone (-4, -3, -2, -1) and two aglycon binding sites ($+1$, $+2$). The binding modes of the benzylidene derivatives indicated a favourable interaction between the Bnl group and subsite (-3) and an unfavourable one with subsite (-4). PPA exhibited a unique pattern of action on CNP-maltooligosaccharides by cleaving maltotriose units from the nonreducing ends and leaving CNP-glycosides, or by cleaving CNP-G_2 units from the reducing ends to leave maltooligosaccharides. Modification of the nonreducing end of NP glycosides to give a 4,6-O-benzylidene-D-glucopyranosyl group indicated a favourable interaction between the Bnl group and the subsites (-3) and (-5) but an unfavourable one with subsite (-4), which resulted in a clear shift in the product pattern. The binding region is longer in BLA than in human amylases. Our results suggested the presence of at least eight subsites; five glycone binding sites and three aglycon ones. The binding modes of substrates will be discussed on the basis of the known features of the structures of α-amylases.

Key words: human salivary α-amylase; porcine pancreatic α-amylase; *Bacillus licheniformis* α-amylase; action patterns; β-maltooligosaccharide glycosides; chemoenzymatic syntheses.

Abbreviations: BLA, *Bacillus licheniformis* α-amylase; Bnl-NP, 4,6-O-benzylidene-modified 4-nitrophenyl-; CD, cyclodextrin; CNP, 2-chloro-4-nitrophenyl-; HPA, human pancreatic α-amylase; HSA, human salivary α-amylase; NP, 4-nitrophenyl-; PPA, porcine pancreatic α-amylase.

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Introduction

α-Amylases α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are classical calcium-containing enzymes that constitute a family of endo-amylases catalysing the cleavage of α-D-(1-4) glycosidic bonds in starch and related carbohydrates with retention of the α-anomeric configuration in the products. They can be found in microorganisms, plants and higher organisms, where they play a dominant role in carbohydrate metabolism (KURIKI & IMANAKA, 1999).

In humans, α-amylase is one of the major secretory products of the pancreas and salivary glands, playing a role in digestion of starch and glycogen. Human α-amylases of both salivary and pancreatic origin, human salivary α-amylase (HSA) and human pancreatic α-amylase (HPA), have been extensively studied from the viewpoint of clinical chemistry, because they are important indicators of pancreatic and salivary glands disorders (e.g. acute pancreatitis, parotitis).

Our interest was focused on salivary amylase, which is a multifunctional enzyme that may play a significant role in dental plaque formation and the subsequent process of dental caries formation and progression (RAMASUBBU et al., 1996).

Furthermore, α-amylases are used as targets for drug design in attempts to treat diabetes, obesity and hyperlipemia. The widening interest in the treatment of sugar metabolic disorders has stimulated our work to search for new and efficient drugs and apply them as inhibitors of amylolytic enzymes. Therefore, we started biochemical studies involving small substrates to gain a better understanding of HSA function.

α-Amylases and related amylolytic enzymes are among the most important enzymes and of great significance in present day biotechnology. They could be potentially useful in semisynthetic chemistry for the formation of oligosaccharides by transglycosylation (CHITRADON et al., 2000).

The spectrum of amylase application has widened in starch saccharification and in the textile, food, brewing and distilling industries. Traditionally, starch hydrolysis was carried out using acid and high temperature (SHILDNECK & SMITH, 1967). Enzymatic hydrolysis of starch has now replaced acid hydrolysis in over 75% of starch-hydrolysing processes due to many advantages, not least its higher yields (FOGARTY, 1983). Hydrolysis of starch gives rise to small maltooligosaccharides and glucose. Saccharide composition obtained after amylolysis of starch is highly dependent on the effect of temperature (MARCHAL et al., 1999), the conditions of hydrolysis and the origin of enzyme. Specificity, thermostability and pH response of the enzymes are critical properties for industrial use.

Bacillus licheniformis is a mesophilic bacterium, but produces a highly thermostable *Bacillus licheniformis* α-amylase (BLA) (DECLERCK et al., 2000). It is widely used in industry for the initial hydrolysis of starch to dextrins, which are then converted to glucose by glucoamylases. However, its function on starch and oligosaccharides is poorly understood. Therefore, we were encouraged to study this α-amylase, and it turned out that it was an attractive model enzyme for active centre investigation.

Material and methods

Substrates

The homologous maltooligomer substrate series (DP 4-8) were synthesised from cyclodextrins by the method of FARKAS et al. (1997). The shorter and longer chain length of CNP-maltooligosides in the range of DP 4-6 and DP 8-11 were prepared by a chemoenzymatic procedure using rabbit skeletal muscle glycogen phosphorylase b (KANDRA et al., 1999, 2001).

Enzymes

α-Amylase (EC 3.2.1.1) from human saliva (Type IX A) was purchased from SIGMA, but isozymes have not been separated. α-Amylase (EC 3.2.1.1) from porcine pancreas (E. Merck) was studied without separation of isozymes. α-Amylase (EC 3.2.1.1) from *Bacillus licheniformis* (Type XII-A, SIGMA) was used without further purification.

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 different temperatures (37 °C for porcine pancreatic α-amylase (PPA) and HSA, 50 °C for BLA) for 5, 7 and 10 min. The reactions were initiated by the addition of 10 μM enzyme to the digest containing 1.7 mM substrate. Samples were taken at the indicated time intervals and the reaction was stopped by the injection of the samples on the chromatographic column. In these studies we have taken care to exclude transglycosylation and secondary attacks on the substrates.

Chromatographic analysis

For HPLC a Hewlett-Packard 1090 Series II Liquid Chromatograph equipped with a diode array detector, automatic sampler, and ChemStation was used. The samples were separated on Supelco NH_2 5 μm column (20 × 0.46 cm) and RP-18 10 μm (20 × 0.46 cm) with different ratios of acetonitrile-water as the mobile phase flowing at a rate of 1 mL/min at 40 °C. Effluent was monitored for NP/CNP groups at 302 nm and the products of the hydrolysis were identified by using relevant standards. The quality of the acetonitrile was

gradient grade. The purified water was obtained from a laboratory purification system equipped with both ion-exchange and carbon filters (Millipore, Bedford, MA, USA).

Mass spectrometry

MALDI-TOF MS analyses of the compounds were performed in positive-ion mode using a Bruker Biflex MALDI-TOF mass spectrometer equipped with delayed-ion extraction. Desorption/ionization of the sample molecules was effected with a 337 nm nitrogen laser with a pulse width of 3 ns. Spectra from multiple (at least 100) laser shots were summarised using 19 kV accelerating and 20 kV reflectron voltages. External calibration was applied using the $[M+Na]^+$ peaks of maltooligosaccharides DP 3-7 and maltoheptaose peracetate, m/z: 527.15, 689.21, 851.26, 1013.31, 1175.36 and 2142.84, respectively. The spectrum was obtained from a 2,4,6-trihydroxyacetophenone matrix by mixing 10 μL of saturated matrix solution with 10 μL of sample dissolved in water, then 0.5 μL was applied to the sample target and was allowed to dry at room temperature. The identification of compounds was done on the basis of the mass of $[M+Na]^+$ peaks.

Results and discussion

Enzymological interest – "Subsite models"

Maltooligosaccharides in the range of maltotriose to maltohexaose have been used as research and clinical reagents as substrates to study the mode of action of α-amylases and, when coupled to a chromogenic aglycon, they form highly sensitive substrates for the detection of α-amylases (Lorentz, 1983; Winn-Deen et al., 1988).

The active sites of depolymerases, and especially of such endoglycanases as α-amylases, are considered to be composed of tandem subsites geometrically complementary to several glucose residues. To examine the active site of an enzyme, X-ray crystallographic analysis of the complex of an enzyme and its substrate analogue is a powerful method (Qian et al., 1997). Many structural studies of PPA have been described, both free and in complex with oligosaccharides (Al Kazaz et al., 1998). In addition, there are structures of HPA and its complexes with analogues (Brayer et al., 2000). There is, however, an X-ray analysis of HSA performed only in the absence of oligosaccharides (Ramasubbu et al., 1996). At the three-dimensional level, the crystal structure of BLA has also been described, together with the structure of calcium-depleted BLA (Machius et al., 1998, 1995). In spite of the extensive studies concerning the structure, thermal properties and molecular mechanism of irreversible thermoinactivation (Violet & Meunier, 1989), little attention has been paid to its enzymological investigation.

The use of modified, low-molecular-weight substrates should therefore be an effective way to elucidate the number of subsites in the active site of HSA and BLA.

Substrates of amylases: chemical synthesis of modified maltooligosaccharides

In the course of our studies of convenient substrates for α-amylases, 2-chloro-4-nitrophenyl- (CNP), 4-nitrophenyl- (NP), and 4,6-O-benzylidene-modified 4-nitrophenyl- (Bnl-NP) β-maltooligosaccharides (DP 3-8) were synthesised and investigated. For the synthesis of these β-maltooligosaccharide glycosides a classical chemical synthesis was developed, based on the use of cyclodextrins. Cyclodextrins (α-, β-, and γ-CDs), prepared on an industrial scale, contain six, seven and eight α-(1-4) bonded glucopyranosyl units, respectively. The conversion of these cyclic oligosaccharides into linear maltooligosaccharides and maltooligosaccharide β-glycosides (DP 3-8) was carried out in our laboratory.

One-pot acetylation and subsequent partial acetolysis of α-, β-, and γ-cyclodextrins resulted in crystalline peracetylated malto-hexaose, -heptaose and -octaose, respectively. Prolonged acetolysis of β-cyclodextrins gave a mixture of acetylated maltooligosaccharides, from which peracetylated malto-triose, -tetraose and -pentaose were isolated. The acetylated oligosaccharides were converted into α-acetobromo derivatives and then transformed into NP- and CNP- β-glycosides. From the NP-glycosides, 4,6-O-benzylidene derivatives were prepared, which were used together with the free glycosides as model substrates to investigate the action pattern and cleavage frequencies of PPA. Figure 1 shows the steps of the synthesis. The detailed procedure, and the separation, purification and structural confirmation of the intermediates and end-products are published (Farkas et al., 1997).

Substrates of amylases: chemoenzymatic synthesis of modified maltooligosaccharides

We describe a new chemoenzymatic procedure of the synthesis of CNP-maltooligosides as a promising alternative to their multistep chemical synthesis, using rabbit skeletal muscle glycogen phosphorylase b (Kandra et al., 1999, 2001).

Detailed enzymological studies revealed that the conversion of CNP-G_7 was highly dependent on the conditions of phosphorolysis and/or transglycosylation. Analysis of reaction products was in-

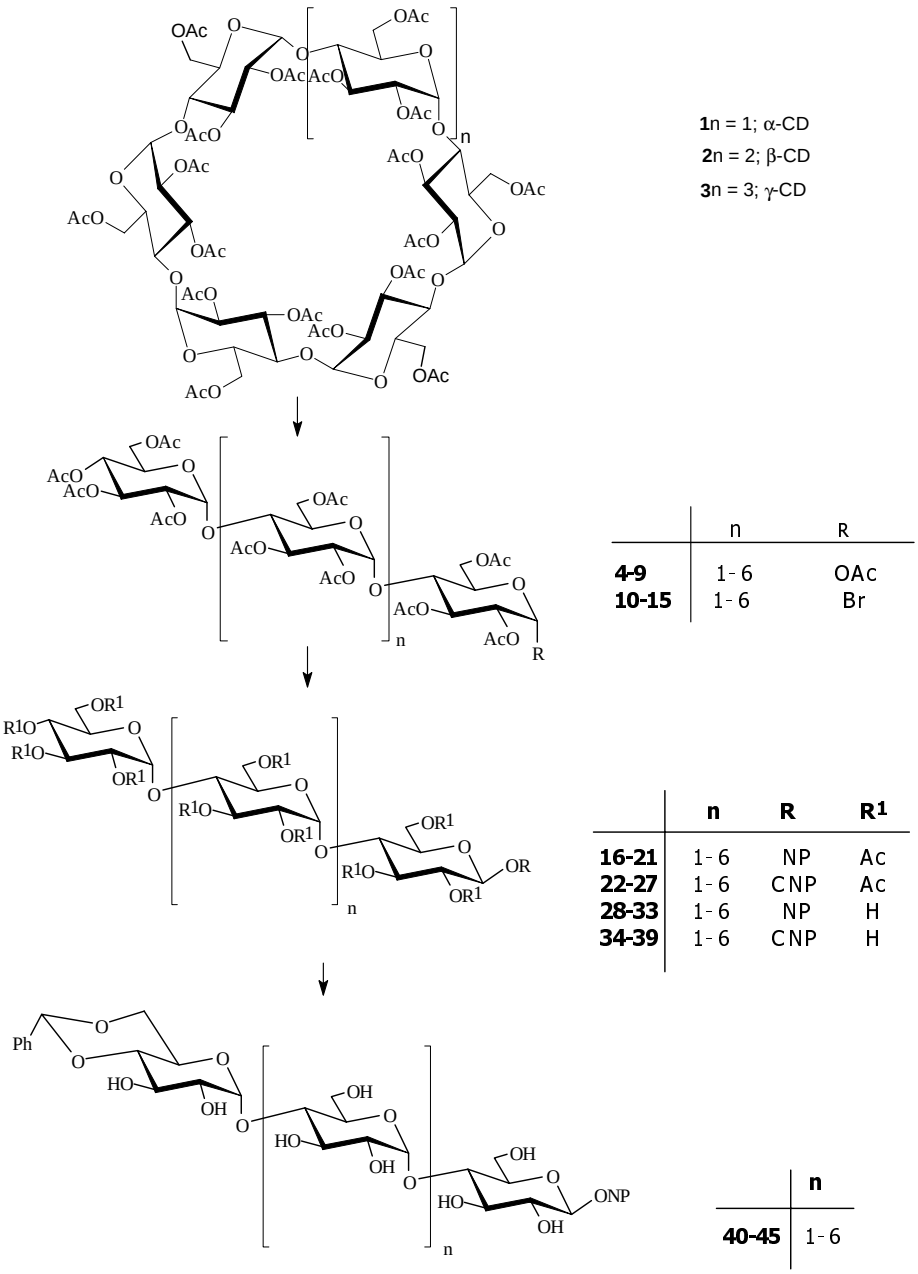


Fig. 1. Synthesis of chromogenic substrates of α-amylases from cyclodextrins. The numbers in bold mean the numbers of synthesized compunds in the work by Farkas et al. (1997).

vestigated using an HPLC system. Figure 2 shows the enzymatic strategy of chain shortening and the HPLC profile of phosphorolysis products. Figure 3 represents the transglycosylation reaction for the preparation of longer oligomers and the MALDI-TOF MS of the elongated products.

The preparative scale isolation of the short (DP 3-6) and longer (DP 8-12) oligomers was achieved by size exclusion chromatography and by HPLC, respectively, on a semipreparative column. The productivity of the syntheses was improved by yields up to 75%. The structures of oligomers were

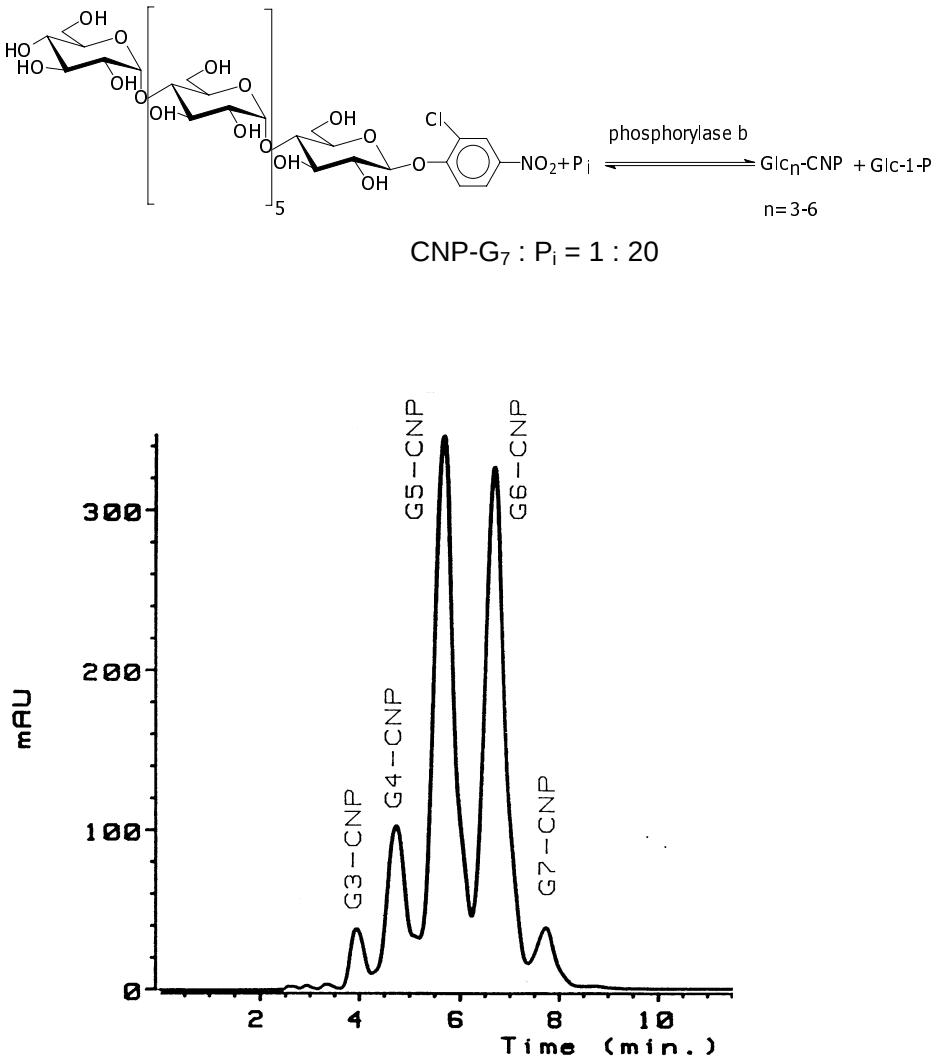


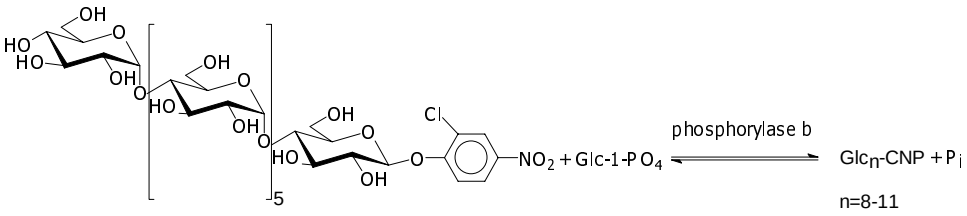
Fig. 2. Chemoenzymatic preparation of 2-chloro-4-nitrophenyl β-maltooligosaccharide glycosides using glycogen phosphorylase b (P_i inorganic phosphate), and HPLC profile of phosphorolysis products.

confirmed by their chromatographic behaviour and MALDI-TOF MS data.

"Action pattern" studies by product-analysis

The application of homologous oligomeric substrates is an effective way to explore the nature of the binding site and the process of catalysis for depolymerising enzymes. Although the overall structures and tertiary folding of polypeptide chains of α-amylase have been described, less is known about the differences in the mode of action of these large depolymerases on the homologous maltooligosaccharide series, and the role of the subsites is poorly understood.

Therefore, our series of substrates were envisaged as good candidates for further studies of the action pattern of PPA, HSA and BLA. These series are unique since neither their preparation, nor their use in the mapping of the active centre of α-amylases has been reported yet. The β-linkage is stable and not hydrolysed by α-amylases; therefore the products of hydrolysis remained UV-detectable. In addition, the 4,6-O-benzylidene-modified oligomers are useful to monitor the digestion products modified at their nonreducing end.



Acceptor:donor = 1 : 10

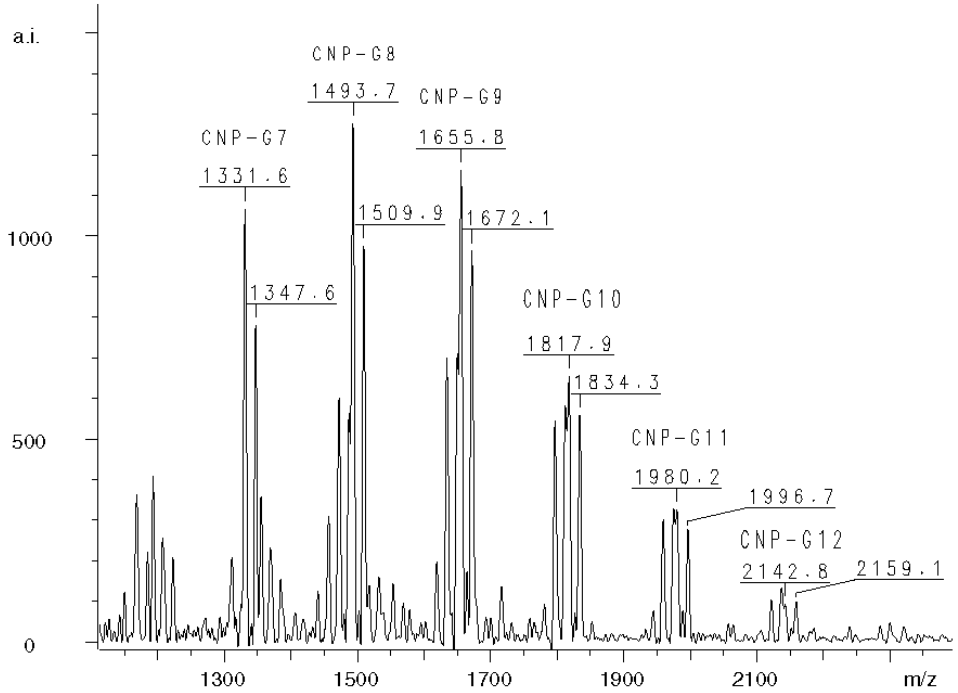


Fig. 3. Chemoenzymatic synthesis of 2-chloro-4-nitrophenyl derivatives of oligosaccharides longer than G_7 using glycogen phosphorylase b, and MALDI-TOF MS of CNP-oligomers. $[M+Na]^+$ peaks correspond to the CNP derivatives of oligosaccharides of DP 7-12.

In this way, the presence or absence of multiple attack can be studied. The hydrolysis products and the remaining substrates were separated and quantified by HPLC, and the columns and elution conditions were selected accordingly. Separation of CNP- and NP-glycosides and their products, produced by amylase, was very effective on an amino column (Fig. 4), since the separated hydrolysis products were members of the same substrate series. However, the benzylidene-modified substrate representatives of the different maltooligosaccharide series (Bnl-NP- and NP-glycosides and 4,6-O-benzylidene-oligosaccharides) could not be separated successfully on an amino column, because of overlapping with members of different series. Therefore, the analysis was carried out on a C18 column, which resulted in good separation (Fig. 5).

Action pattern of HSA: evidence for a "six subsite model" (KANDRA & GYÉMÁNT, 2000)

Our results revealed that the binding region in

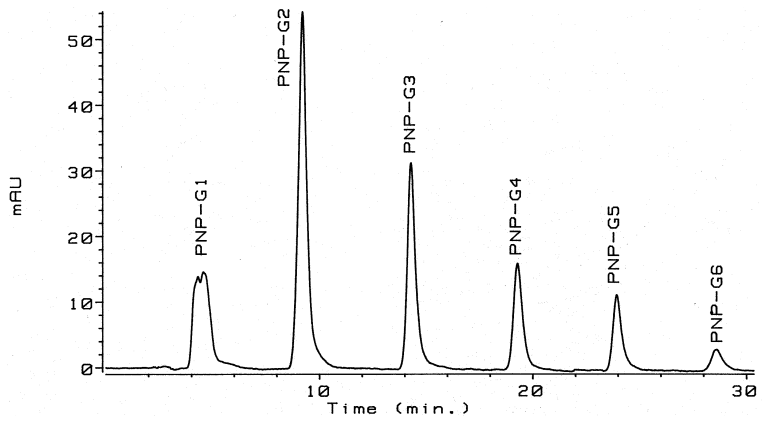
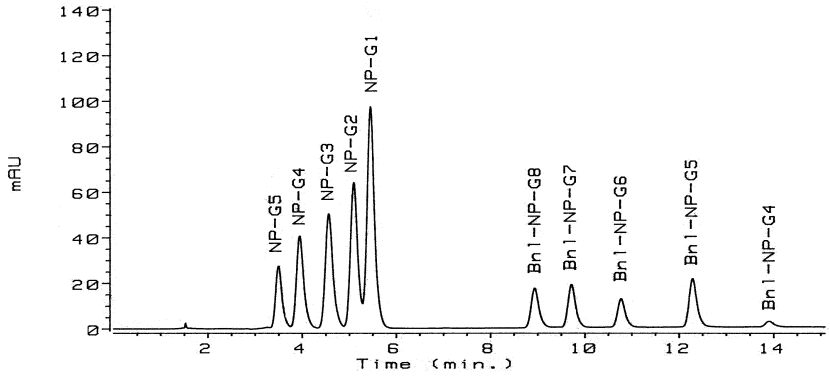
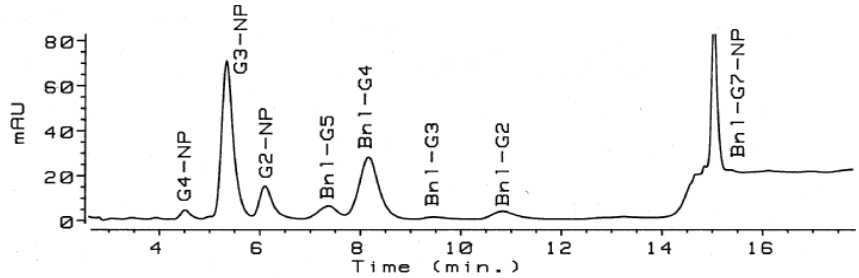


Fig. 4. Separation of PNP (p-nitrophenyl)-maltooligosides on an amino column by HPLC using acetonitrile-water gradient elution.



(a)



(b)

Fig. 5. HPLC separation of 4,6-O-benzylidene-modified NP-oligosaccharides and their hydrolysis products on a C18 column by gradient elution. (a) Substrates and NP hydrolysis products. (b) 4,6-O-benzylidene-4-nitrophenyl maltoheptaoside and its NP- and benzylidene-modified hydrolysis products.

HSA is longer than the five subsites usually considered in the literature, and suggested the presence of at least six subsites: four glycone binding sites (-4, -3, -2, -1) and two aglycon binding sites ($+1$, $+2$). When a substrate is not long enough to occupy all six subsites, hydrolysis results in the prominent formation of CNP-G_2 (85% and 86% from CNP-G_4 and CNP-G_5,

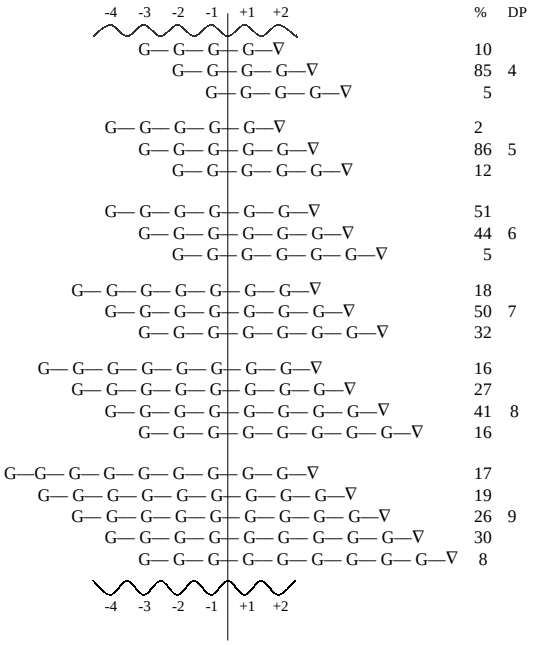


Fig. 6. Schematic representation of CNP-oligosides binding to subsites of HSA. Bond cleavage frequencies are expressed as percentages of total cleavage events. G, glucosyl residue; ∇, 2-chloro-4-nitrophenyl group.

respectively). In the ideal arrangement, the six subsites are filled by a glucopyranosyl unit and the release of maltotetraose from the nonreducing end is dominant (CNP-G_6). In the case of the longer oligomers (CNP-G_7 – CNP-G_9), release of maltotetraose from the nonreducing ends of the substrates remains predominant. The binding modes of the benzylidene derivatives indicated a favourable interaction between the Bnl group and subsite (−3), and an unfavourable one with subsite (−4). Schematic representation of CNP- and benzylidene-modified NP-glycosides binding to subsites of HSA can be seen in Figure 6 and Figure 7, respectively.

Action pattern of PPA: the popular "five-subsite model" (KANDRA et al., 1997)

PPA exhibited a unique pattern of action on CNP-maltooligosaccharides by cleaving the maltotriose units from the nonreducing ends and leaving CNP-glycosides, or by cleaving CNP-G_2 units from the reducing ends to leave maltooligosaccharides. Full amylase activity could be obtained only when the five subsites were occupied and these observations were consistent with the popular "five-subsites model" (ROBYT & FRENCH, 1970).

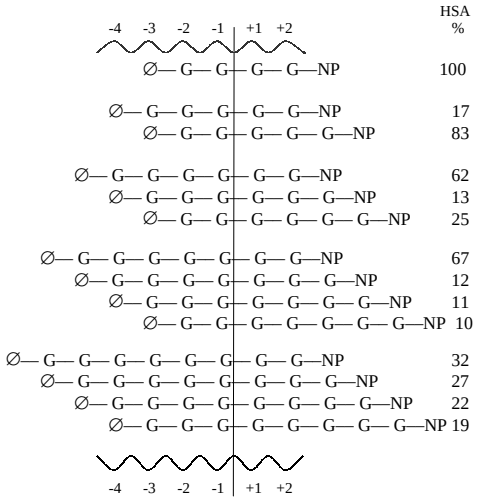


Fig. 7. Schematic representation of 4,6-O-benzylidene-modified NP-oligosaccharides binding to subsites of HSA. G, glucosyl residue; Ø, benzylidene group; NP, 4-nitrophenyl group.

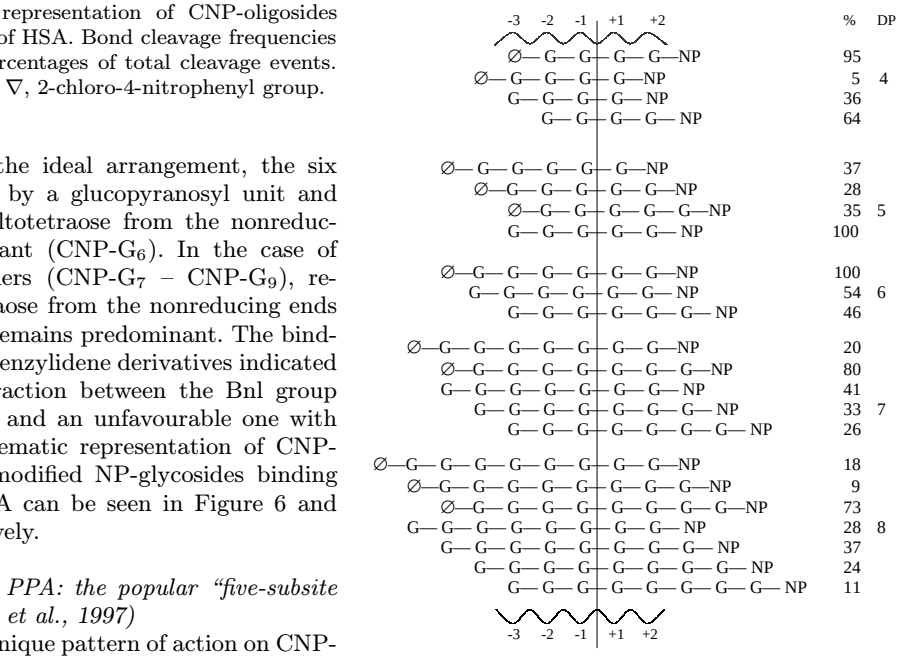


Fig. 8. Schematic representation of substrates binding to subsites of PPA. G, glucosyl residue; NP, 4-nitrophenyl group; Ø, benzylidene group.

Modification of the nonreducing end of NP-glycosides to give a 4,6-O-benzylidene-D-glucopyranosyl group indicated a favourable interaction

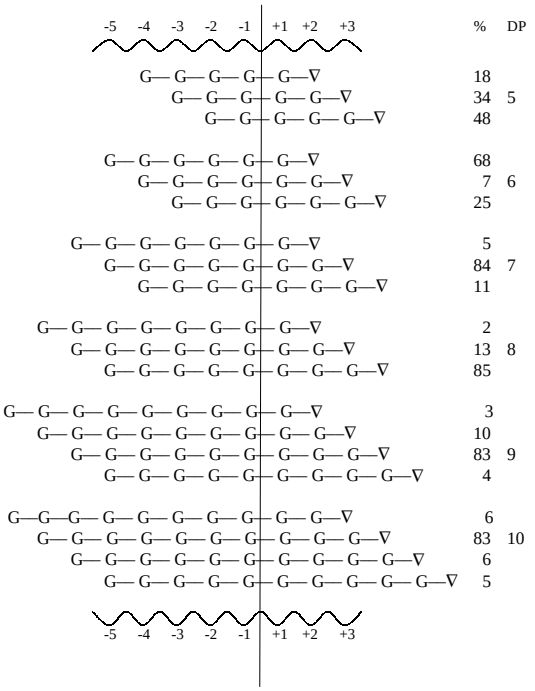


Fig. 9. Action pattern of BLA on CNP-maltooligosaccharide β-glycosides. G, glucosyl residue; ∇, 2-chloro-4-nitrophenyl group.

between the Bnl group and subsites (−3) and (−5), but an unfavourable one with subsite (−4) which resulted in a clear shift in the product pattern. Figure 8 shows the schematic representation of substrates binding to subsites of PPA.

Action pattern of BLA: eight subsites are suggested
The action pattern of BLA was studied by paper chromatography using ^{14}C-reducing-end-labelled maltooligosacharides. The cleavage frequency of the glycosidic bonds has, however, not been quantified (Nakakuki et al., 1984).

The action pattern and product specificity of BLA were examined by utilising as model substrates CNP β-glycosides of maltooligosaccharides of DP 5-10 (prepared by a chemoenzymatic route), and two NP derivatives modified at the nonreducing end with a benzylidene group.

The results revealed that the binding region in BLA was longer than those of human amylases and suggested the presence of at least eight subsites: five glycone (−5, −4, −3, −2, −1) and three aglycon binding sites (+1, +2, +3). In the ideal arrangement, the eight subsites are filled by a glucopyranosyl unit. The cleavage of maltopentaose from the nonreducing end is dominant for the shorter substrates (DP 6-8). In the case of the longer substrates (DP 8-10), however, the release of CNP/NP-G_3 from the reducing end becomes preferred. This interesting dual product-specificity of BLA could be observed on the Bnl-modified substrates as well, which resulted in the dominant formation of Bnl-G_5 or G_3-NP.

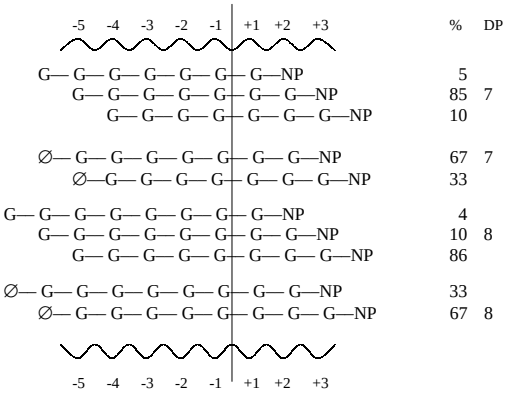


Fig. 10. Action pattern of BLA on NP-glycosides and their benzylidene-protected derivatives. G, glucosyl residue; NP, 4-nitrophenyl group; Ø, benzylidene group.

Figures 9 and 10 show the action pattern of BLA on maltooligosaccharide glycosides. The action pattern and product specificity of BLA has not been examined so far because of the difficulties of the synthesis of higher-molecular-weight oligosaccharide glycosides with chromogenic aglycons.

These results confirm that our series of substrates are indispensable tools in the investigation of the binding sites of α-amylases altered by protein engineering.

Acknowledgements

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References

Al Kazaz, M., Desseaux, V., Marchis-Mouren, G., Prodanov, E. & Santimone, M. 1998. The mechanism of porcine pancreatic α-amylase. Inhibition of maltopentaose hydrolysis by acarbose, maltose and maltotriose. Eur. J. Biochem. **252:** 100–107.

Brayer, G. D., Sidhu, G., Maurus, R., Rydberg, E. H., Braun, C., Wang, Y., Nguyen, N. T., Overall, C. M. & Withers, G. S. 2000. Subsite mapping of the human pancreatic α-amylase active site through structural, kinetic, and mutagenesis techniques. Biochemistry **39:** 4778–4791.

Chitradon, L., Mahakhan, P. & Bucke, C. 2000. Oligosaccharide synthesis by reversed catalysis using α-amylase from *Bacillus licheniformis*. J. Mol. Catal. **B10:** 273–280.

Declerck, N., Machius, M., Wiegand, G., Huber, R. & Gaillardin, C. 2000. Probing structural determinants specifying high thermostability in *Bacillus licheniformis* α-amylase. J. Mol. Biol. **301:** 1041–1057.

Farkas, E., Jánossy, L. Harangi, J., Kandra, L. & Lipták, A. 1997. Synthesis of chromogenic substrates of α-amylases on a cyclodextrin basis. Carbohydr. Res. **303:** 407–415.

Fogarty, W.M. 1983. Microbiol enzymes and biotechnology, pp. 1–92. In: Fogarty, W.M. (ed.) Applied Science Publishers Ltd., London.

Kandra, L. & Gyémánt, G. 2000. Examination of the active sites of human salivary α-amylase (HSA). Carbohydr. Res. **329:** 579–585.

Kandra, L., Gyémánt, G. & Lipták, A. 1997. Action pattern of porcine pancreatic α-amylase on three different series of β-maltooligosaccharide glycosides. Carbohydr. Res. **298:** 237–242.

Kandra, L., Gyémánt, G. & Lipták, A. 1999. Chemoenzymatic preparation of 2-chloro-4-nitrophenyl β-maltooligosaccharide glycosides using glycogen phosphorylase b. Carbohydr. Res. **315:** 180–186.

Kandra, L., Gyémánt, G., Pál, M., Petró, M., Remenyik, J. & Lipták, A. 2001. Chemoenzymatic synthesis of 2-chloro-4-nitrophenyl β-maltoheptaoside acceptor-products using glycogen phosphorylase b. Carbohydr. Res. **333:** 129–136.

Kuriki, T. & Imanaka, T. 1999. The concept of the α-amylase family: structural similarity and common catalytic mechanism. J. Biosci. Bioeng. **87:** 557–565.

Lorentz, K. 1983. Evaluation of α-amylase assays with 4-nitrophenyl-α-oligosaccharides as substrates. J. Clin. Chem. Clin. Biochem. **7:** 463–471.

Machius, M., Wiegand, G. & Huber, R. 1995. Crystal structure of Ca-depleted *Bacillus licheniformis* α-amylase at 2.2 Å resolution. J. Mol. Biol. **246:** 545–559.

Machius, M., Declerck, N., Huber, R. && Wiegand, G. 1998. Activation of *Bacillus licheniformis* α-amylase through a disorder-order transition of the substrate-binding site mediated by a calcium-sodium-calcium metal triad. Structure **15:** 281–292.

Marchal, L. M., Van De Laar, A. M. J., Goetheer, E., Schimmelpennink, E. B., Bergsma, J., Beeftink, H. H. & Tramper, J. 1999. Effect of temperature on the saccharide composition obtained after α-amylolysis of starch. Biotechnol. Bioeng. **63:** 344–355.

Nakakuki, T., Azuma, K. & Kainuma, K. 1984. Action patterns of various exo-amylases and the anomeric configurations of their products. Carbohydr. Res. **128:** 297–310.

Qian, M., Spinelli, S., Driguez, H. & Payan, F. 1997. Structure of a pancreatic α-amylase bound to a substrate analogue at 2.03 Å resolution. Protein Sci. **6:** 2285–2296.

Ramasubbu, N., Paloth, V., Luo, Y., Brayer, G. D. & Levine, M. J. 1996. Structure of human salivary α-amylase at 1.6 Å resolution: implications for its role in the oral cavity. Acta Cryst. **D52:** 435–446.

Robyt, J. F. & French, D. 1970. Multiple attack and polarity of action of porcine pancreatic α-amylase. Arch. Biochem Biophys. **138:** 662–670.

Shildneck, P. & Smith, C. E. 1967. Starch: chemistry and technology, pp. 217–235. In: Whistler, R. L. & Paschall, E. F. (eds) Academic Press, New York.

Violet, M. & Meunier, J.-C. 1989. Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α-amylase. Biochem. J. **263:** 665–670.

Winn-Deen, E. S., David, H., Sigler, G. & Chavez, R. 1988. Development of a direct assay for α-amylase. Clin. Chem. **10:** 2005–2008.

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