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Action pattern of porcine pancreatic alpha-amylase on three different series of β-maltooligosaccharide glycosides

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Abstract

A technique for the investigation of the action pattern of porcine pancreatic amylase (PPA) has been developed by utilising as model substrates 2-chloro-4-nitrophenyl (CNP) and 4-nitrophenyl (NP) β-glycosides of maltooligosaccharides of dp 4–8 and some NP derivatives modified at the nonreducing end with a 4,6-*O*-benzylidene (Bnl) group. The action pattern was investigated by the method of product analysis, using an HPLC method. The product pattern and cleavage frequency was very similar in the CNP- and NP-oligomers and showed that the glucopyranose residue could be replaced by the aglycon group. 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 results obtained with the digestion of the benzylidene-protected substrates confirm a multiple attack mechanism for PPA.

Keywords: Porcine pancreatic alpha-amylase; Action pattern; Maltooligosaccharides; HPLC

1. Introduction

Clinical importance.—Measuring alpha-amylase (EC 3.2.1.1) activity in serum, urine, and other biological fluids is a useful laboratory screening test for the diagnosis of pancreatic disorders [1]. Several methods have been reported, which are based on different principles using such substrates as limit dextrin [2], modified starch [3], maltooligosaccharides [4,5], or maltooligosaccharide derivatives containing 4-nitrophenyl (NP) [6–9] or 2-chloro-4-nitrophenyl (CNP) [10–12] as aglycon groups.

Recently some blocked substrates carrying a modified residue at the nonreducing end have also been reported, and it has been shown that such groups as ethylidene [13], 3-oxobutylidene [14], benzylidene [15], benzyl [16], and galactopyranosyl [17] protect the substrates from hydrolysis by exo-glucosidases which are used as coupling enzymes in the alpha-amylase assays.

In the course of our studies of convenient substrates for alpha-amylase, 2-chloro-4-nitrophenyl (CNP), 4-nitrophenyl (NP), and 4,6-*O*-benzylidene-

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modified 4-nitrophenyl (Bnl-NP) β-maltooligosaccharides (dp 4 to 8) were synthesised and investigated. Application of defined substrates of low molecular weight was preferred because the purity of these substances and their reaction patterns can be exactly determined.

Enzymological significance.—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 structure and the tertiary folding of the polypeptide chain of porcine pancreatic alpha-amylase (PPA) have been determined [18], less is known about the differences in the action of PPA on the homologous maltooligosaccharide series. Therefore, our substrate series were envisaged as good candidates for further studies of the action pattern of PPA. Compared with other substrate series so far reported, for example, maltooligosaccharides [19] or α-NP-maltooligosaccharides [20], the CNP- and Bnl-NP-maltooligosaccharides, which are β-glycosides, are unique since neither their preparation nor their use in the mapping of the active centre of alpha-amylases have been reported. This β linkage is stable and is not hydrolysed by alpha-amylases. In addition, the 4,6-*O*-benzylidene-modified oligomers are very stable towards hydrolysis by exo-glucosidases and are useful for monitoring the digestion products modified at the nonreducing end. In this way the presence or absence of a multiple attack can be studied. Selection of these glycosides as substrates was based on their size and good yields when synthesised from cyclodextrins. It is important to note that these compounds are highly water-soluble.

The present studies were aimed at determining the relationship between the action pattern of PPA and the mode of binding of these three different glycoside series to PPA by using an HPLC method. In addition, the effect of aglycons and the role of the benzylidene group at the nonreducing end on the action pattern of PPA were examined. The results are discussed on the basis of the known features of the structure of PPA, assuming that the active centre of PPA includes five subsites [19]. An attempt has been made to employ this theory to explain semiquantitatively the differences observed in the pattern of action on maltooligosaccharide derivatives.

2. Results

Action pattern and cleavage frequencies of PPA on CNP-maltooligosaccharides.—A series of CNP-maltooligosaccharides (dp 4 to 8) was used in the PPA reaction to determine unambiguously the exact glycosidic linkage being cleaved, as well as the cleavage frequency which, indicating the binding mode of the corresponding substrate, was tested by HPLC on an aminopropylsilica (APS) column. Only the chromogenic reaction products could be detected with a diode array detector (DAD) at 302 nm, and therefore these compounds were quantified. The concentrations of glycosidic fragments produced by amylase 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 Table 1.

PPA exhibits 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. As the chain length increases, the concentration of the main product (CNP-G_2) decreases; in the case of the octamer, the maximum frequency of attack shifts from the reducing end into the inside of the chain and CNP-G_3 becomes the major product. These results are consistent with the five-subsite model of PPA.

Patterns of action and cleavage frequencies of PPA on NP-maltooligosaccharides and their 4,6-O-benzylidene-blocked derivatives (Bnl-NP).—A series of 4-nitrophenyl-β-maltooligosaccharides (dp 4–8) and their derivatives blocked at the nonreducing end with a 4,6-*O*-benzylidene group were also hydrolysed with PPA. Only the 4-nitrophenyl glycosides formed were quantified, and the results are given in Table 2 as an average of at least six determinations. The distribution of the products from the benzylidene-pro-

Table 1
Yields [a] of products from the hydrolysis of CNP-maltooligosaccarides by PPA

Substrate	Products (mol/mol of 2-chloro-4-nitrophenyl products) [b]				
	G_1-CNP	G_2-CNP	G_3-CNP	G_4-CNP	G_5-CNP
G_4-CNP	0.38	0.62			
G_5-CNP	tr. [c]	1.00			
G_6-CNP		0.54	0.46		
G_7-CNP		0.44	0.34	0.22	
G_8-CNP		0.25	0.40	0.25	0.10

[a] The yields given are an average of at least six determinations.
[b] Experimental error is ±5% of values given.
[c] tr., Traces.

tected substrates was analysed on an RP18 column, as well.

The product pattern of the 4,6-*O*-benzylidene oligomers was very interesting and markedly different from that of the unmodified substrates. From the hexamer glycoside only one prominent glycosidic fragment, G_2-NP, was released. From the heptamer two (G_2-NP and G_3-NP) and from the octamer three (G_2-NP, G_3-NP, and G_4-NP) glycosidic products were released. Three cleavages occur in the benzylidene-blocked NP-pentoside with about the same frequency yielding G_1-NP, G_2-NP, and G_3-NP. These unexpected and surprising results will be discussed later in section 3. Fig. 1 shows that the profiles of NP-glucosides that result from the hydrolysis of 4-nitrophenyl glycosides and their benzylidene-modified derivatives differ significantly.

Multiple attack by PPA on 4,6-O-benzylidene-modified substrates.—Taking advantage of the fact that benzylidene-blocked hydrolysis products can be monitored at 200 nm by HPLC on an RP18 column, the product distribution during digestion of the $BnlG_7$-NP, catalysed by PPA, was quantitated at intervals at the optimum pH 6.9, where multiple attack occurs. If the substrate is cleaved only once in the absence of a repetitive attack, then the concentration of G_2-NP must equal that of $BnlG_5$ and the concentration of G_3-NP must equal that of $BnlG_4$. However, a repetitive attack on the substrate will tend to decrease the concentration of the larger oligomers and increase the concentration of the smaller ones.

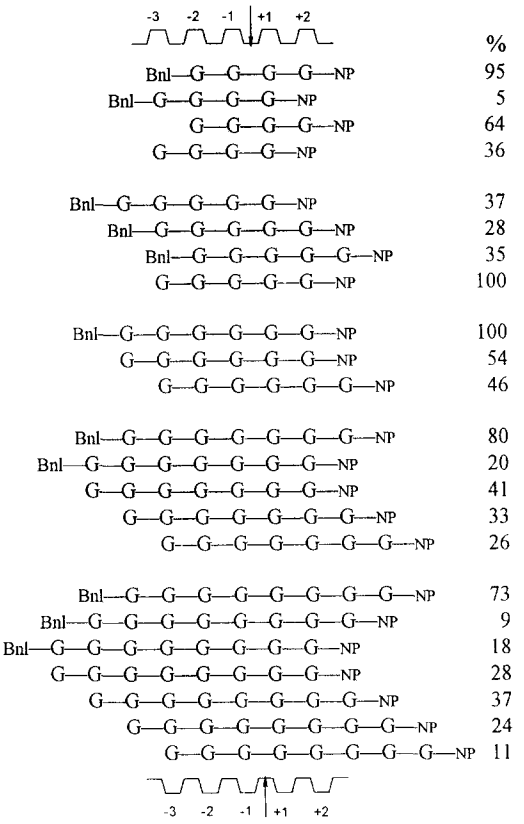

Fig. 1. Schematic representation of substrate(s) binding to subsites of PPA. Bond-cleavage frequencies are expressed as percentages of total cleavage events calculated from the reaction products. G, glucosyl residues; NP, 4-nitrophenyl groups (connected to the reducing end in β form); ——— represents linkages; Bnl, benzylidene group; ↓, catalytic site [situated between subsites (−1) and (+1)].

Table 2
Yields of products from hydrolysis of NP- and 4,6-*O*-benzylidene-protected NP-maltooligosaccharides by PPA

Substrate	Column	Products (mol/mol of 4-nitrophenyl products) [a]				
		G_1-NP	G_2-NP	G_3-NP	G_4-NP	G_5-NP
G_4-NP	NH_2	0.36	0.64			
$BnlG_4$-NP	NH_2	0.05	0.95			
	RP18	0.05	0.95			
G_5-NP	NH_2		1.00			
$BnlG_5$-NP	NH_2	0.37	0.28	0.35		
	RP18	0.39	0.25	0.36		
G_6-NP	NH_2		0.54	0.46		
$BnlG_6$-NP	NH_2		1.00	tr. [b]	tr.	
	RP18		0.97	0.02	0.01	
G_7-NP	NH_2		0.41	0.33	0.26	
$BnlG_7$-NP	NH_2		0.27	0.73	tr.	
	RP18		0.20	0.80		
G_8-NP	NH_2		0.28	0.37	0.24	0.11
$BnlG_8$-NP	NH_2		0.18	0.09	0.73	
	RP18		0.20	0.10	0.70	

[a] Experimental error is ±5% of values given.
[b] tr., Traces.

Table 3
Yields of modified products from the early stages of hydrolysis of 4,6-*O*-benzylidene-G_7-NP by PPA on an RP18 column

Conversion (%)	Products (mol/mol) [a]					
	G_3-NP	G_2-NP	$BnlG_5$	$BnlG_4$	$BnlG_3$	$BnlG_2$
2.9	0.795	0.215	0.156	0.735	0.028	0.081
5.7	0.805	0.195	0.161	0.725	0.027	0.087
8.6	0.805	0.195	0.161	0.730	0.028	0.081

[a] Experimental error is ±5% of values given.

We found that the equal concentration of G_2-NP and $BnlG_5$ and the equal concentration of G_3-NP and $BnlG_4$ disappeared eventually, and the analysis of the degradation products, modified at the reducing end and nonreducing end, gave evidence of repetitive attack by PPA. In this study we have taken care to exclude a secondary attack on the substrate. The product ratios were always obtained from the early stages of hydrolysis (conversion < 10%), before any secondary attack could be detected. The results are shown in Table 3.

3. Discussion

All of the enzymes so far examined in the 'alpha-amylase family' are multidomain proteins and their catalytic domain folds in the form of a $(\beta/\alpha)_8$-barrel [21]. The active site of these enzymes can be described in terms of contiguous subsites, where a subsite is a section of the active site that interacts with one monosaccharide residue of the substrate. From enzymological studies, PPA is believed to have five subsites (subsite −3 to +2) and the catalytic site is located between the second (+1) and the third (−1) subsites from the reducing end [19]. There are a number of different ways in which a substrate can interact with the subsites. In the present study only the productive complexes have been considered. Analysis of the hydrolysis products reveals which subsites of the active centre are occupied in a given binding mode. For the shorter substrates (G_4), the NP and CNP moieties would be equivalent to a glucose residue as suggested by MacGregor et al. [9] and found by Ajandouz and Marchis-Mouren [20]. It was found that the more favourable arrangement was the binding mode 3 (bm 3) where two glucosyl residues from the reducing end of the tetramers were bound in subsites (+1) and (+2), which resulted in the release of CNP/NP-G_2. If three glucosyl units were fixed from the nonreducing end of the tetramers in subsites (−3), (−2), and (−1), monomer glycosides (CNP/NP-G_1) could be formed (bm 2). In these cases the aglycons (CNP/NP), which were in β-glycosidic linkages, could interact with subsite (+2) of the substrate binding region, but less favourably than a glucose residue (data not shown). However, this is not true with the longer substrates (G_5–G_8). In the ideal arrangement all subsites were filled by glucosyl units; thus, from the pentamers only one productive complex could be formed, and this resulted in one glycosidic fragment, CNP/NP-G_2 (bm 3). CNP/NP-G_6 was cut almost equally to CNP/NP-G_2 (bm 3) and CNP/NP-G_3 (bm 4). Three internal bonds were hydrolysed in the heptaoside, producing CNP/NP-G_2 (bm 3), CNP/NP-G_3 (bm 4), and CNP/NP-G_4 (bm 5), in a decreasing order. Four internal bonds were cut in the octaoside, but the major cut led to the liberation of CNP/NP-G_3 indicating that the favourable binding mode was 4 (bm 4). Our results agreed with those reported by Seigner et al. [23] in that full amylase activity could be obtained only when the five subsites were occupied, and our observations were consistent with the five-subsite model.

The number of binding modes of 4,6-*O*-benzylidene-protected oligomers was markedly different from that of the unmodified ones. It was envisaged that the Bnl group would not mimic a glucopyranosyl unit, but we found that it was recognisable by subsite (−3). The hydrolysis products of $BnlG_4$-NP were 95% G_2-NP, while those from G_5-NP were 100% G_2-NP. Hydrolysis of $BnlG_5$-NP resulted in 35% G_3-NP compared to 0% G_3-NP when G_5-NP was hydrolysed. These suggest a favourable interaction between the Bnl group and subsite (−3). On the other hand, hydrolysis of $BnlG_5$-NP, G_6-NP, and G_5-NP resulted in 28, 54, and 100% of G_2-NP, respectively, indicating that there was a low affinity between the Bnl group and subsite (−4). These results can be explained by the three-dimensional structural model of the PPA–acarbose complex described by Qian et al. [18]. If subsite (−4) is occupied by the 4,6-*O*-benzylidene residue, this will cause steric hindrance because of Trp59, therefore leading to a decreased affinity. Conversely, the Bnl group at subsite (−5) increased the affinity, assuming a favourable interaction with subsite (−5), Therefore, the site of hydrolysis was moved closer to the reducing end, which resulted in a clear shift in the action pattern. This tendency is most evident for the longer substrates (G_6–G_8) where all subsites are occupied by glucopyranosyl residues, but also exists in the case

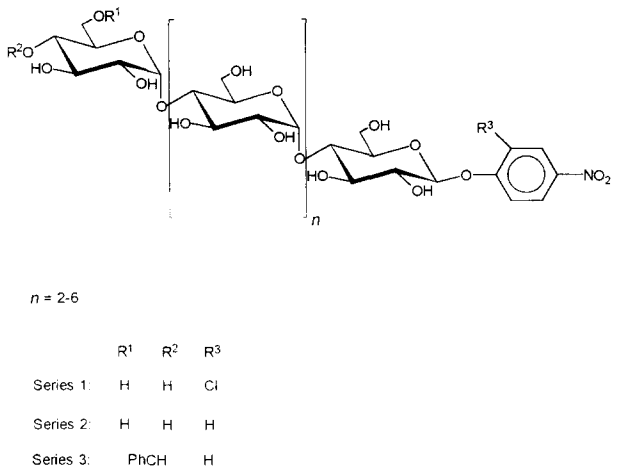

Fig. 2. Schematic representation of the substrates of PPA.

of the shorter ones (G_5 and G_4). It can be assumed that both ends of the active site contain hydrophilic regions which are not advantageous to the apolar residues. Comparison with the popular model for the catalytic action of porcine-amylase, proposed by Robyt and French [19], indicates that the 4,6-*O*-benzylidene portion of the molecule must have very specific structural characteristics if it is to mimic the binding of glucosyl residues in the PPA active site.

Although a multiple attack is well-documented for mammalian alpha-amylases [24], there is no evidence that it is important in the action on short oligosaccharides. Our results on $BnlG_7$-NP are consistent with the reports that PPA exhibits a repetitive attack.

4. Experimental

Substrates.—The homologous maltooligomer substrate series 1, 2, and 3 (dp 4–8) were synthesised by the method of Lipták et al. (unpublished data). Fig. 2 shows the structure of the substrate series.

Enzyme.—Alpha-amylase (EC 3.2.1.1) from porcine pancreas was purchased from E. Merck, but isozymes I and II have not been separated. Ishizuka et al. [22] reported cleavage distribution of maltooligosaccharides catalysed by PPA II with results close to those of Robyt and French [19] using a mixture of both amylase isozymes.

Hydrolysis of the maltooligosaccharides.—Incubations in 50 mM HEPES buffer (pH 6.9) containing 5 mM $CaCl_2$, 50 mM NaCl, and 3 mM NaN_3 were carried out at 37 °C for 2, 4, 6, and 8 min. The reactions were initiated by the addition of 10 μM enzyme to the digest containing 0.5 mM substrate. Samples were taken at the indicated time intervals

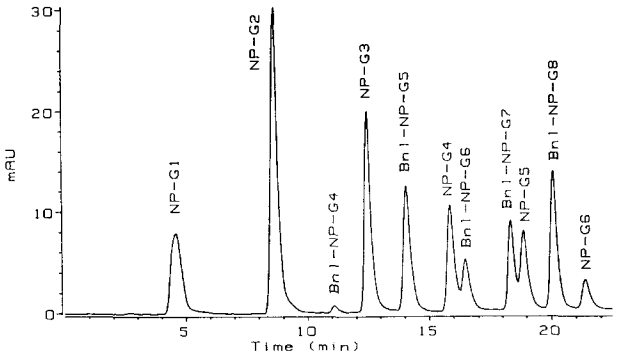

Fig. 3. Separation of 4,6-*O*-benzylidene-protected NP oligosaccharides and their NP-hydrolysis products on an APS column by HPLC.

and the reaction was stopped by the injection of the samples on the chromatographic column.

Chromatographic analysis.—For HPLC a Hewlett–Packard 1090 series II Liquid Chromatograph equipped with a diode array detector, automatic sampler, and ChemStation was used. Each sample was separated both on a Hewlett–Packard APS 10-μm column (0.46 × 20 cm) and a Hewlett–Packard RP18 10-μm column (0.46 × 20 cm) with different ratios of CH_3CN–water as the mobile phases flowing at a rate of 1.0 mL/min at 40 °C. Effluent was monitored for NP/CNP and Bnl groups at 302 nm and 200 nm, respectively, and the products of the hydrolysis were identified by using relevant standards. The quality of the acetonitrile was HPLC grade. Purified water was obtained from a laboratory purification system equipped with both ion-exchange and carbon filters (Millipore, Bedford, MA, USA).

Product analysis and quantitation.—The hydrolysis products and remaining substrates were separated and quantitated by HPLC, and the columns and elution conditions were selected accordingly. Separation

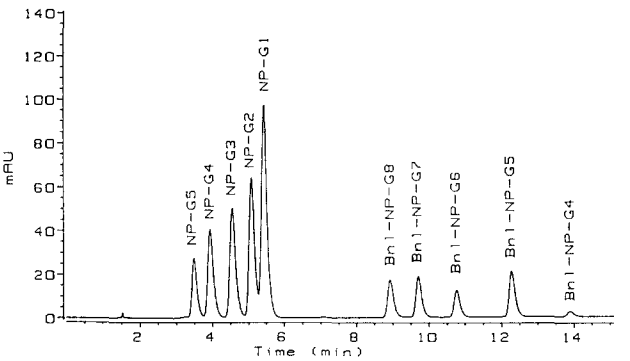

Fig. 4. Separation of 4,6-*O*-benzylidene-modified NP oligosaccharides and their NP-hydrolysis products by HPLC on an RP18 column.

Table 4
Parameters of HPLC analysis

Substrates and products	Column	Eluents (MeCN–water)
G_{1-8}-NP	NH_2	0 min 88:12; 20 min 60:40
	RP18	15:85
G_{1-8}-CNP	NH_2	0 min 88:12; 20 min 60:40
	RP18	15:85
$BnlG_{4-8}$-NP	NH_2	0 min 90:10; 18 min 60:40
	RP18	0 min 15:85; 10 min 30:70

of CNP- and NP-glucosides and their products, produced by amylase, was very effective on an APS column since the separated hydrolysis products were the members of the same substrate series. However, the benzylidene-modified substrate representatives of the three different maltooligosaccharide series (Bnl-NP- and NP-glucosides, and 4,6-*O*-benzylidene-oligosaccharides) could not be separated successfully on an ASP column because of overlapping with the members of different series (Fig. 3). Therefore, the analysis was carried out on an RP18 column, which resulted in a good separation (Fig. 4). Table 4 shows the optimum chromatographic conditions for separation.

Acknowledgements

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