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Carbohydrate Research 329 (2000) 579–585

CARBOHYDRATE RESEARCH

Examination of the active sites of human salivary α-amylase (HSA)

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Received 9 June 2000; accepted 24 July 2000

Dedicated to Professor András Lipták on the occasion of his 65th birthday

Abstract

The action pattern of human salivary amylase (HSA) was examined by utilising as model substrates 2-chloro-4-nitrophenyl (CNP) β-glycosides of maltooligosaccharides of dp 4–8 and some 4-nitrophenyl (NP) derivatives modified at the nonreducing end with a 4,6-*O*-benzylidene (Bnl) group. The product pattern and cleavage frequency were investigated by product analysis using HPLC. The results revealed that the binding region in HSA is longer than 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 aglycone binding sites ($+1$, $+2$). In the ideal arrangement, the six subsites are filled by a glucosyl unit and the release of maltotetraose (G_4) from the nonreducing end is dominant. The benzylidene group was also recognisable by subsites (-3) and (-4). 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). Thus, subsite (-4) must be more hydrophylic than hydrophobic. As compared with the action of porcine pancreatic α-amylase (PPA) on the same substrates, the results showed differences in the three-dimensional structure of active sites of HSA and PPA.

Keywords: Human salivary α-amylase; Substrate specificity; Maltooligosides; HPLC separation

1. Introduction

Clinical importance.—α-Amylase (EC 3.2.1.1) is one of the major secretory products of the pancreas and salivary glands in humans, playing a role in digestion of starch and glycogen. Human α-amylases of both salivary and pancreatic origin (HSA and HPA, respectively) have been extensively studied from the view point of clinical chemistry because they are important as indicators of dysfunction of the tissue of origin. The pancreas and salivary glands have amylase concentrations that are several orders of magnitude greater than that of other normal tissue, and these two organs probably account for almost all of the serum amylase activity in normal persons. Measuring α-amylase activity in serum, urine, saliva and other biological fluids is a useful diagnostic tool in evaluating diseases of the pancreas and salivary glands (e.g., acute pancreatitis, parotitis) [1,2].

A variety of methods are now available that are based on different principles using such substrates as limit dextrins [3], modified starch [4], maltooligosaccharides [5,6], or maltooligosaccharide derivatives containing 4-

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0008-6215/00/$ - see front matter
PII: S0008-6215(00)00221-4

nitrophenyl (NP) [7–10], or 2-chloro-4-nitrophenyl (CNP) [11–13] as aglycon groups. 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 [14], 3-oxobutylidene [15], benzylidene [16], benzyl [17], and galactopyranosyl [18] protect the substrates from hydrolysis by exo-glucosidases, which are used as coupling enzymes in the α-amylase kits. The method for α-amylase, with 2-chloro-4-nitrophenyl-α-maltotrioside as substrate, requires no helper enzymes and is suitable for routine assay of clinical specimens [19].

In the course of our studies of convenient substrates for α-amylase, 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 [20]. For the preparation of short chain length CNP-maltooligosides in the range dp 3–6, a new chemoenzymatic procedure was developed using rabbit skeletal muscle glycogen phosphorylase b [21]. Fig. 1 shows the structure of this substrate series.

Enzymological significance.—The homologous maltooligosaccharide substrates are indispensable tools in the investigation of the binding site and the action of different depolymerising enzymes. In these studies well defined, high purity, low-molecular-weight substrates are preferred because purity and their reaction patterns can be exactly determined. Therefore, our substrate series were envisaged as good candidates for further studies of the action pattern of HSA. Compared with other substrate series so far reported, for example, maltooligosaccharides [22] or α-NP-maltooligosaccharides [23], the CNP- and Bnl-NP-maltooligosaccharides are β-glycosides. Their preparation and their use in the mapping of the active centre of PPA was first reported by our laboratory [24]. The β linkage is stable and is not hydrolysed by α-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.

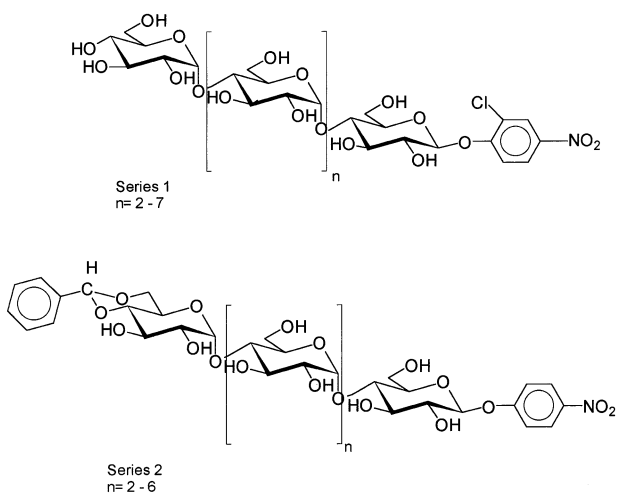

Fig. 1. Structure of the substrates of HSA.

Present studies were aimed at determining the relationship between the action pattern of human salivary amylase (HSA) and the mode of binding of the CNP and Bnl-NP glycoside series to HSA by product analysis 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 HSA were examined. These results are discussed on the basis of the subsite concept, introduced by Phillips and his co-workers in the study of lysozyme and Schechter and Berger in their work on proteinase papain [25].

2. Results

Action pattern and cleavage frequencies of HSA on CNP-maltooligosaccharides.—A series of CNP-maltooligosaccharides (dp 4–9) was used in the HSA reaction to determine unambiguously the exact glycosidic linkage being cleaved, as well as the cleavage frequency tested by high-performance liquid chromatography (HPLC) on an aminopropylsilica (NH_2) 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.

Table 1
Yields [a] of products from the hydrolysis of CNP-maltooligosides by HSA

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_6-CNP
G_4-CNP	0.10	0.85	0.05			
G_5-CNP	0.02	0.86	0.12			
G_6-CNP		0.51	0.44	0.05		
G_7-CNP		0.18	0.50	0.32		
G_8-CNP		0.16	0.27	0.41	0.16	
G_9-CNP		0.17	0.19	0.26	0.30	0.08

[a] The yields given are an average of at least four determinations.
[b] Experimental error is ± 5% of the values given.

HSA exhibits an interesting pattern of action on CNP maltooligosaccharides by cleaving the maltose (85%) and maltotriose (86%) units as the main products from the nonreducing ends of CNP-G_4 and CNP-G_5, respectively, and leaving CNP-G_2 glycosides. In the case of the hexamer glycoside, the maximum frequency of attack shifts from the nonreducing end to the inside of the chain and the maltotetraose (G_4) becomes the major product. The cleavage of the maltotetraose from the nonreducing ends of the oligomers remained favourable in the case of the hydrolysis of the heptamer, octamer and nonamer glycosides also, resulting in 50, 41 and 30% of maltotetraose, respectively.

*Patterns of action and cleavage frequencies of HSA on the 4,6-*O*-benzylidene-blocked NP-maltooligosaccharides (Bnl-NP).*—A series of 4-nitrophenyl-β-maltooligosaccharides (dp 4–8) blocked at their nonreducing end with a 4,6-*O*-benzylidene group was hydrolysed with HSA. Only the 4-nitrophenyl glycosides formed were quantified, and the results are given in Table 2 as an average of at least four determinations. The results show that the product patterns of CNP-glycosides and the benzylidene modified NP-glycosides differ significantly.

Only one prominent glycosidic fragment, G_2-NP was released from the tetramer glycoside. From the pentamer two (G_2-NP and G_3-NP) and from the hexamer three (G_2-NP, G_3-NP and G_4-NP) glycosidic products were formed. Four cleavages occur in the longer benzylidene blocked substrates, yielding G_2-NP, G_3-NP, G_4-NP and G_5-NP glycosides.

These interesting and unexpected results will be discussed later in Section 3.

3. Discussion

Human α-amylases are divided into two groups by their site of biosynthesis. One group is the pancreatic α-amylase and the other is the salivary α-amylase. Their amino acid sequences have been deduced from the nucleotide sequences of cDNAs [26], and that of HSA was also determined from the cloned gene [27]. These sequences are quite similar, with 97% homology between the two α-amylases. Each contains several isozymes, which have been separated by isoelectric focusing and polyacrylamide gel electrophoresis [28]. Human saliva contains more than five α-amylase isozymes [29]. There are some differences in the physico-chemical properties of these isozymes, but their active sites may be identical.

Table 2
Yields of products from the hydrolysis of 4,6-*O*-benzylidene-protected NP-maltooligosides by HSA

Substrate	Products (mol/mol of 4-nitrophenyl products) [a]			
	G_2-NP	G_3-NP	G_4-NP	G_5-NP
BnlG_4-NP	1.00			
BnlG_5-NP	0.17	0.83		
BnlG_6-NP	0.62	0.13	0.25	
BnlG_7-NP	0.67	0.12	0.11	0.10
BnlG_8-NP	0.32	0.27	0.22	0.19

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

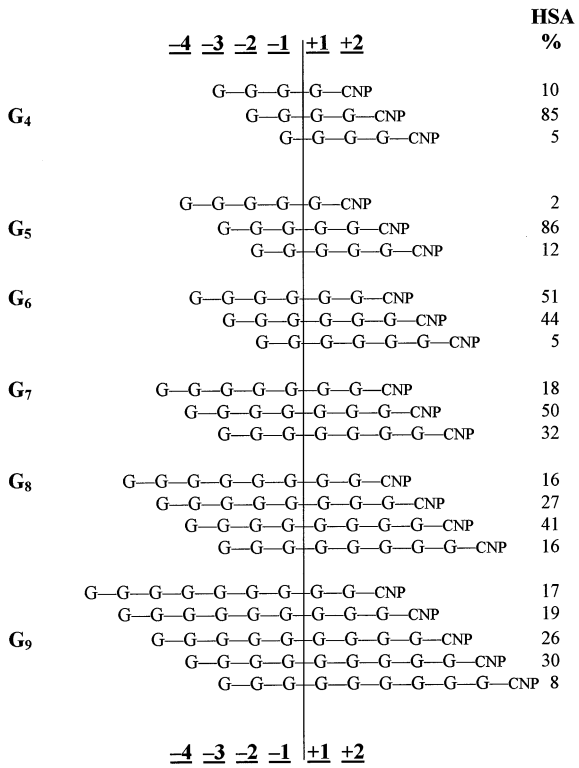

Fig. 2. Schematic representation of CNP-glycosides binding to subsites of HSA. Bond cleavage frequencies are expressed as percentages of total cleavage events calculated from the reaction products. G, glucosyl residues; CNP, 2-chloro-4-nitrophenyl groups (connected to the reducing end in β form); (—) linkages; (↓) catalytic site.

The active sites of depolymerases and especially of such endoglycanases as HSA are estimated 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. Many structural studies of PPA have been described, both free and in complex with oligosaccharides [30]. In addition there are structures of the more relevant HPA and its complexes with analogues [31]. However, there is only an X-ray analysis of the human salivary amylase [32] performed in the absence of oligosaccharides. The use of modified, low-molecular-weight substrates should therefore be an effective way to elucidate the number of subsites in the active site area of HSA.

From enzymological studies, human salivary amylase is believed to have five subsites, three glycone binding sites and two aglycone binding sites [33], there is no third subsite for the aglycone binding site [34].

We have studied the active site of HSA by examination of its modes of action on CNP β-maltooligosides (Fig. 2) and modified NP β-maltooligosides, which had 4,6-*O*-benzylidene groups at their nonreducing ends (Fig. 3).

It has been found that all CNP β-maltooligosaccharide substrates have more than one bond cleaved; three cleavages occur in the case of CNP-G_4, CNP-G_5, CNP-G_6 and CNP-G_7 oligomers and four glycosidic bonds were hydrolysed in CNP-G_8. For the shorter substrates (G_4 and G_5) the CNP moiety would be equivalent to a glucose residue, as was found for PPA in our previous work [21]. In these cases the aglycons (CNP), which were in β-glycosidic linkages, could interact with subsite (+2), but less favourably than a glucose residue. In the ideal arrangement subsite (+2) was filled by a glycopyranosyl unit and the hydrolysis resulted in the prominent formation of CNP-G_2 (85% from CNP-G_4 and 86%

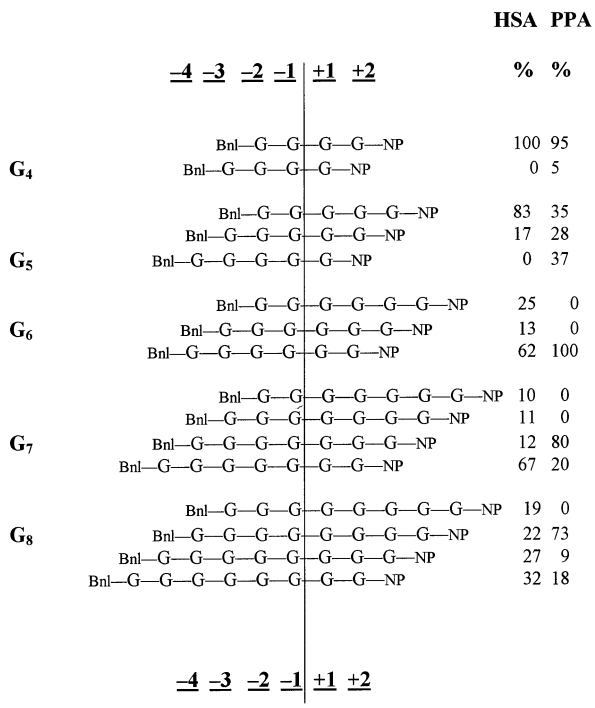

Fig. 3. Schematic representation of benzylidene-modified NP-glycosides binding to subsites of HSA and PPA. Bond cleavage frequencies are expressed as percentages of total cleavage events calculated from the reaction products. G, glucosyl residues; NP, 4-nitrophenyl groups (connected to the reducing end in β form); Bnl, benzylidene groups; (—) linkages; (↓) catalytic site.

from CNP-G_5). This favourable release of CNP-G_2 was also observed for the hexamer glycoside (CNP-G_6), where four glycosyl residues from the nonreducing end were bound in subsites (-4, -3, -2, -1) and led to the formation of maltotetraose in a 51% yield. In the case of the longer substrates (CNP-G_7, CNP-G_8 and CNP-G_9) release of maltotetraose from the nonreducing ends of the substrates remains predominant resulting in 50, 41 and 30% of CNP-G_3, CNP-G_4 and CNP-G_5, respectively.

Our results show that the full amylase activity could be obtained when the six subsites (-4, -3, -2, -1, $+1$, $+2$) were occupied and these experiments are consistent with a six-subsite model, which had been proposed for PPA by Gilles and co-workers in 1996 [30].

The number of binding modes of 4,6-*O*-benzylidene protected oligomers was different from that of the unmodified ones. It was envisaged that the 4,6-*O*-benzylidene group would not mimic a glycopyranosyl unit, but we found that it was recognisable by subsites (-3) and (-4). The hydrolysis product of BnlG_4-NP was 100% BnlG_2, and that from BnlG_5-NP was 83%. These suggest a very favourable interaction between the benzylidene residue and subsite (-3). It is important to mention that the same observations were made by Nagamine and co-workers [35,36] on modified phenyl α-maltooligosides protected with iodo or 2-pyridylamino groups at their nonreducing ends.

As the chain length increases, the concentration of the main product (BnlG_2) decreases; 25, 10 and 0% for the hexamer, heptamer and octamer glycosides, respectively. Surprisingly, there was a low affinity between the 4,6-*O*-benzylidene group and subsite (-4). Formation of BnlG_3 shows a low cleavage frequency for this hydrolysis product; 0, 17, 23, 11 and 19% for the BnlG_4-NP, BnlG_5-NP, BnlG_6-NP, BnlG_7-NP and BnlG_8-NP, respectively. On the other hand, formation of G_2-NP from the reducing ends of glycosides shows a high cleavage frequency for this hydrolysis product; 100, 17, 62, 67 and 32% for BnlG_4-NP, BnlG_5-NP, BnlG_6-NP, BnlG_7-NP, respectively. The relatively low release of G_2-NP (17%) from the pentamer glycoside (BnlG_5-NP) corresponds to a low affinity between the Bnl group and subsite (-4).

In an ideal arrangement two glucose molecules are fixed in the aglycone binding sites (subsites $+1$ and $+2$) of the active center and the BnlG_2 residue is fixed in the glycone binding sites (subsites -3, -2 and -1). However, the action patterns of the non-modified CNP-glycosides suggest that there is a fourth subsite on the glycone binding site (subsite -4), which has a higher affinity for the binding of a glycopyranosyl unit than that of benzylidene group. Therefore, the subsite (-4) must be rather more hydrophylic than hydrophobic.

Our results provide evidence that the binding region in the HSA molecule is longer than five contiguous subsites usually considered in the literature. It is suggested from our studies with HSA on modified maltooligosaccharides that HSA has at least six subsites (-4, -3, -2, -1, $+1$, $+2$) like PPA [30] and that the glycosidic bond split by this enzyme is between subsite (-1) and subsite ($+1$). On the other hand, our results are in general agreement with those obtained for the human pancreatic amylase [31], since a total of five subsites and an additional glycone subsite was proposed in that case. However, the binding modes of the benzylidene derivatives to the active site of salivary α-amylase were found to be different from those of porcine pancreatic α-amylase (Fig. 3). This can be explained by the slight difference in their primary and crystal structure. Namely, the amino acid 163 located in the vicinity of subsite (-3) is valine in the case of porcine pancreatic amylase [30], but it is serine for human salivary amylase [32]. This change in polarity might result in differences in the action pattern of substrates, especially if the benzylidene group is located at position (-4).

Benzylidene derivatives may also elucidate the differences between the active sites of HSA and HPA rather than other modified substrates.

4. Experimental

Substrates.—The homologous maltooligomer substrate series 1, 2 (Fig. 1) (dp

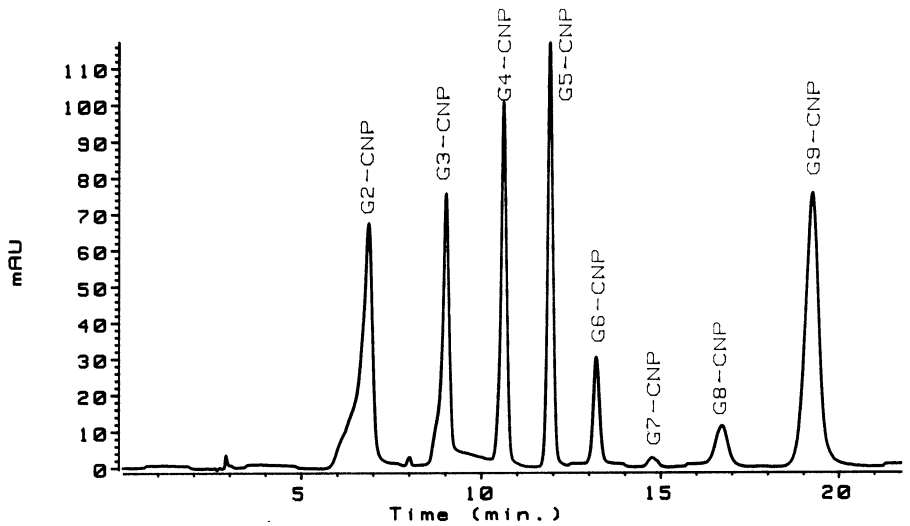

Fig. 4. Separation of CNP-oligomers in the range dp 2–8.

4–8) were synthesised from cyclodextrins by the method of Lipták et al. [20]. The shorter chain length of CNP-maltooligosides in the range dp 3–6 were prepared by a chemoenzymatic procedure, as well as using rabbit skeletal muscle glycogen phosphorylase b [24].

Enzyme.—α-Amylase (EC 3.2.1.1) from human saliva (Type IX A) was purchased from Sigma (Chem. Co. Ltd.) and analysed without separation of the isozymes.

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 37 °C for 5, 7 and 10 min. The reactions were initiated by the addition of 10 μM of enzyme to the digest 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 on the substrates. The product ratios were always obtained from the early stages of hydrolysis (conversion < 10%), before any secondary attack could be detected.

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) with different ratios of MeCN–water as the mobile phase flowing at a rate of 1 mL/min at 40 °C. The 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 MeCN 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). Fig. 4 shows the separation of CNP-oligomers in the range dp 2–8.

Acknowledgements

This work was supported by a grant from the Hungarian Scientific Research Fund (OTKA T 032005).

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