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Note

Modified malto-oligosaccharides as inhibitors of human alpha-amylases

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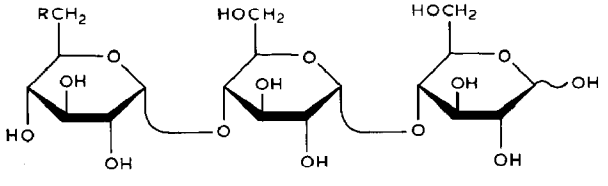
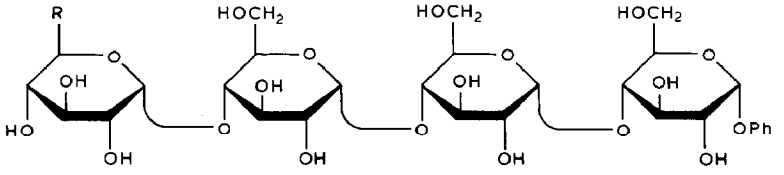
X-ray crystallographic analysis of the complex of an enzyme and an inhibitor (substrate analog) gives information useful for deductions about the interaction between enzyme and substrate, as shown by Phillips and assoc.[1,2] in a study of lysozyme action.

We have studied two human alpha-amalyses, salivary (HSA) and pancreatic alpha-amylases (HPA), which have similar amino acid sequences[3]. Little is known about their active sites[4–6], and their action mechanisms remain to be elucidated. As maltooligosaccharides and their derivatives have been used as inhibitors in the X-ray analysis of Taka-amylase A[7,8] and porcine pancreatic alpha-amylase[9], we tested the inhibition, by the modified maltooligosaccharides **1**–**6**, of the hydrolysis of 4-nitrophenyl α-maltotetraoside by human alpha-amylases.



1 (Glc_3) R = OH
2 ($IGlc_3$) R = I
3 ($BGlc_3$) R = OCH_2Ph
4 ($PGlc_3$) R = $NHC_5H_5N(2)$



5 ($AGlc_4Ph$) R = CH_2NH_2
6 ($GlcAGlc_3Ph$) R = CO_2H

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Maltotriose (**1**, Glc_3) and some of its derivatives, *O*-(6-deoxy-6-iodo-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (**2**, $IGlc_3$), *O*-(6-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (**3**, $BGlc_3$), and *O*-{6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl}-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (**4**, $PGlc_3$), are not hydrolyzed by human alpha-amylases. Two derivatives of phenyl α-maltotetraoside, phenyl *O*- (6-amino-6-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl- (1→4) -*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside (**5**, $AGlc_4Ph$) and phenyl *O*-(α-D-glucopyranosyluronic acid)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside (**6**, $GlcAGlc_3Ph$), are hydrolyzed by these enzymes, but at very low rates as compared with the rate of hydrolysis of phenyl α-maltotetraoside[5,6].

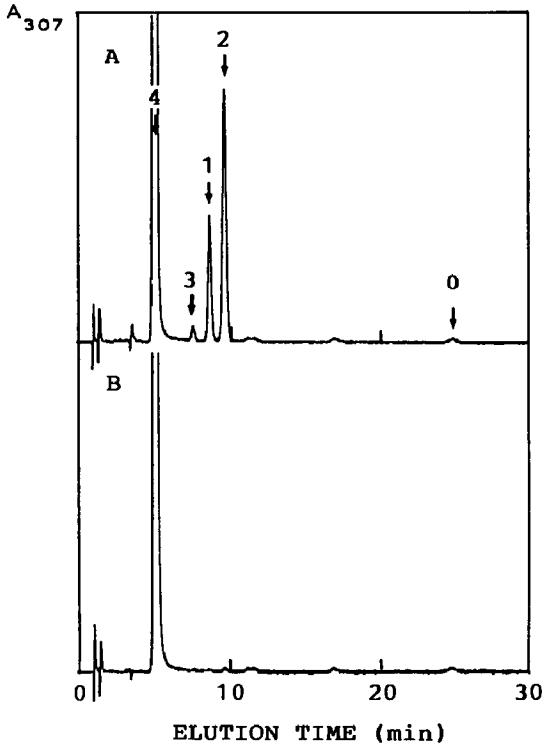

Fig. 1. H.p.l.c. of the digest of 4-nitrophenyl α-maltotetraoside by HSA in the presence of $IGlc_3$ (**2**); (A) After 5 min of digestion; (B) at zero time. The elution positions of standard substances are shown in A by arrows. (0) 4-nitrophenol, (1) 4-nitrophenyl α-D-glucopyranoside, (2) 4-nitrophenyl α-maltoside, (3) 4-nitrophenyl α-maltotrioside, and (4) 4-nitrophenyl α-maltotetraoside.

4-Nitrophenyl α-maltotetraoside was incubated with HSA or HPA in the presence or absence of one of the inhibitors, and each digest was analyzed by h.p.l.c. Fig. 1 shows typical chromatograms. HSA hydrolyzed 4-nitrophenyl α-maltotetraoside to give 4-nitrophenyl α-D-glucopyranoside and maltotriose (29%), 4-nitrophenyl α-maltoside and maltose (68%), and 4-nitrophenyl α-maltotrioside and D-glucose (3%); for

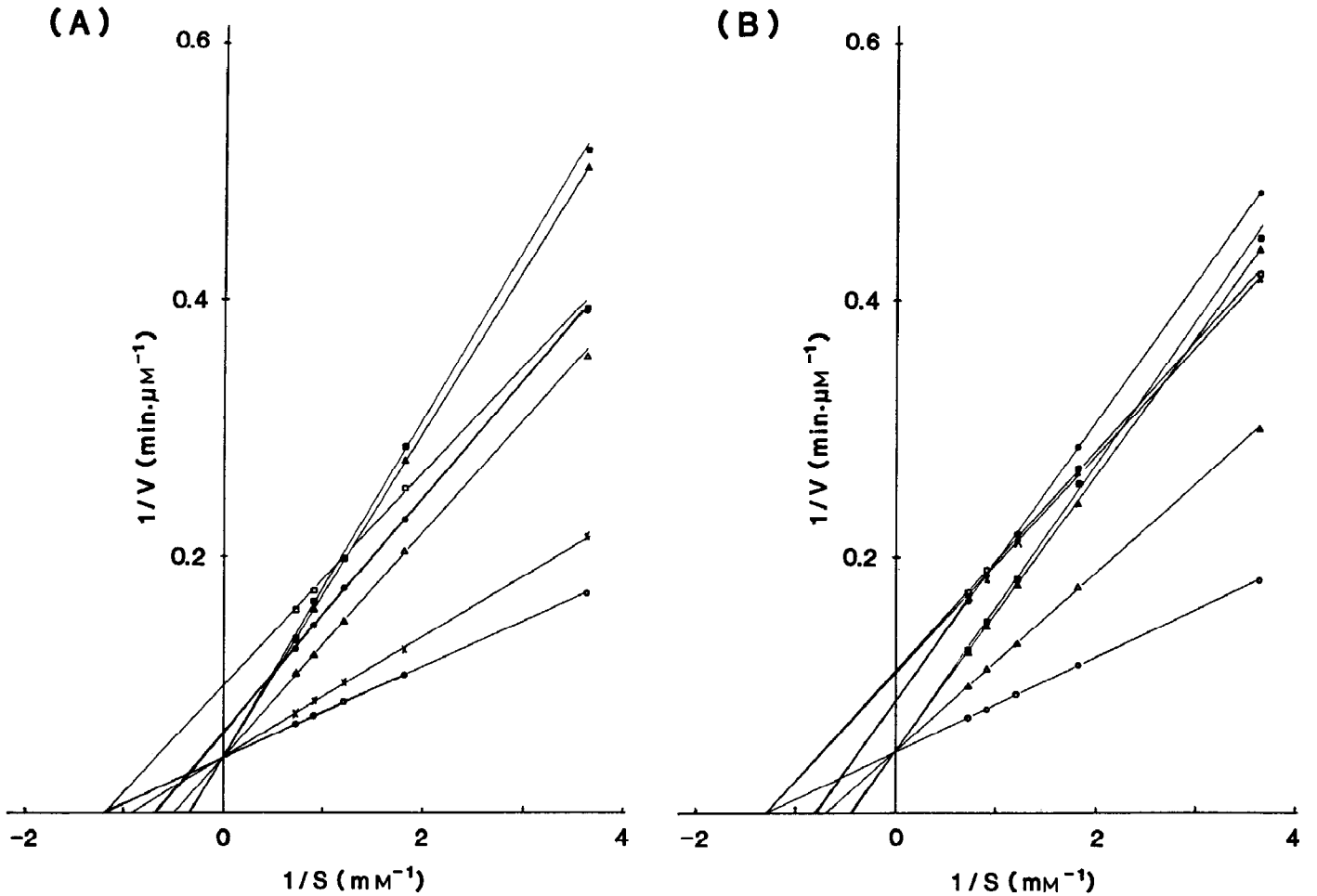

Fig. 2. Lineweaver–Burk plots for the hydrolysis of 4-nitrophenyl α-maltotetraoside catalyzed by HSA and HPA in the presence or absence of an inhibitor. The plotted values of the initial velocities were the means of experiments repeated three times. (A, HSA). (○) Without inhibitor, (●) with 5.6mM Glc_3 (1), (△) with 0.90mM $IGlc_3$ (2), (▲) with 4.6mM $BGlc_3$ (3), (□) with 2.8mM $PGlc_3$ (4), (■) with 4.8mM $AGlc_4Ph$ (5), and (x) with 5.4mM $GlcAGlc_3Ph$ (6). (B, HPA). (○) Without inhibitor, (●) with 5.6mM Glc_3 (1), (△) with 0.90mM $IGlc_3$ (2), (▲) with 4.6mM $BGlc_3$ (3), (□) with 1.4mM $PGlc_3$, (4), (■) with 2.4 mM $AGlc_4Ph$ (5), and (x) with 5.4mM $GlcAGlc_3Ph$ (6).

TABLE I

Inhibition by maltooligosaccharide derivatives of the hydrolysis of 4-nitrophenyl α-maltotetraoside by HSA or HPA at 37° and pH 7.0

Inhibitor	*Type of inhibition*[a]		K_i (mM)	
	HSA	*HPA*	*HSA*	*HPA*
Glc_3 (1)	M	M	3.8	2.9
$IGlc_3$ (2)	C	C	0.83	0.98
$BGlc_3$ (3)	C	C	1.9	2.4
$PGlc_3$ (4)	NC	NC	2.2	1.1
$AGlc_4Ph$ (5)	C	C	1.9	1.2
$GlcAGlc_3Ph$ (6)	C	NC	16	4.1

[a] (C) Competitive inhibition; (NC) noncompetitive inhibition; and (M) mixed-type inhibition.

HPA, these percentages were 45, 48, and 7%, respectively. The initial velocity (v) of hydrolysis was calculated from the total amounts of the products. The type of inhibition was judged from the Lineweaver–Burk plots ($1/v$ *vs.* 1/s, Fig. 2), and the value of K_i was obtained from the ratio of the slopes with and without the inhibitor[10] ($1+i/K_i$). The K_m values of 4-nitrophenyl α-maltotetraoside for HSA and HPA were 0.83 and 0.77mM, respectively. The K_i values obtained are given in Table I. The reason for the different types of inhibition obtained with Glc_3 and its derivatives is not yet clear.

The inhibitor constants of $AGlc_4Ph$ (**5**) were not very different from those of $IGlc_3$ (**2**), although the rates of hydrolysis of $AGlc_4Ph$ (**5**) by the enzymes were much lower than those of phenyl *O*-(6-deoxy-6-iodo-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside[5,6]. This suggested that $AGlc_4Ph$ (**5**) was nonproductively and strongly bound to the active sites of the enzymes. $GlcAGlc_4Ph$ (**6**) is hydrolyzed by the enzymes little if at all[5,6], and its inhibitor constants are very large. It is likely that $GlcAGlc_3Ph$ (**6**) is rarely bound to the active sites. The effect of $GlcAGlc_3Ph$ (**6**) was different on HSA and HPA; thus this inhibitor recognizes differences between the active sites of the two enzymes.

$IGlc_3$ (**2**) was a competitive inhibitor of both enzymes, and its K_i values for the enzymes were the smallest of the inhibitors examined here. The values were low enough to be used in X-ray studies, as judged from the results[7] for Taka-amylase A. In addition, $IGlc_3$ (**2**) contains a heavy atom, iodine, which is a marker for X-ray crystallography. Therefore, it is the most suitable substrate analog of the compounds we tested for X-ray crystallographic study.

EXPERIMENTAL

Materials. — HSA and HPA were purified from saliva and pancreatic juice, respectively, by the method of Matsuura *et al.*[11]. One unit of alpha-amylase is defined as the amount of enzyme that hydrolyzes 1 μmol of 4-nitrophenyl α-maltotetraoside per

min at pH 7.0 and 37°. 4-Nitrophenyl α-maltotetraoside, purchased from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.), was further purified by gel filtration on a Bio-Gel P-2 column (2.5 × 200 cm) equilibrated with 0.05M acetic acid. Glc_3 (**1**) was purified, from a maltotriose preparation donated by Nihon Shokuhin Kako (Tokyo, Japan), on a Bio-Gel P-2 column (2.5 × 200 cm) equilibrated with 0.05M acetic acid. $IGlc_3$ (**2**), $PGlc_3$ (**4**), $AGlc_4Ph$ (**5**), and $GlcAGlc_3Ph$ (**6**) were prepared as described previously[12–14]. $BGlc_3$ (**4**) was prepared by the hydrolysis of 4-nitrophenyl *O*-(6-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside[15] by HSA, followed by gel filtration of the digest as described above.

Measurement of the initial velocity of the enzymic hydrolysis. — A solution (150 μL) of 4-nitrophenyl α-maltotetraoside, an inhibitor, and HSA or HPA in 0.05M *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid–NaOH buffer containing 0.01M $CaCl_2$, pH 7.0, was incubated at 37° for 5 min. The concentration of HSA or HPA was 0.014 unit.ml^{-1} and the substrate concentration was from 0.27 to 1.4mM. M Acetic acid (200 μL) was added to the mixture to stop the enzymic reaction. A portion of the digest was analyzed by h.p.l.c. for determination of the amount of each product. The initial velocity was calculated from the total amount of each product.

H.p.l.c. — The h.p.l.c. apparatus was equipped with a Shimadzu pump Model 6 AD, a Hitachi UV monitor Model 638, an SIC Chromatocorder 11, and a column of Wakosil 5C18 (4.6 x 150 mm; Wako Pure Chemicals, Osaka, Japan). The column was eluted with 0.05M ammonium acetate buffer, pH 4.5, containing 1% butanol at the flow rate of 1.5 mL.min^{-1}. Elution was monitored at 307 nm for detection of the 4-nitrophenyl residue.

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