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Carbohydrate Research 331 (2001) 13–18

CARBOHYDRATE RESEARCH

Inhibition of β-glycosidases by acarbose analogues containing cellobiose and lactose structures

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Received 6 November 2000; received in revised form 8 January 2001; accepted 9 January 2001

Abstract

Acarbose analogues, containing cellobiose and lactose structures, were prepared by reaction of the two disaccharides with acarbose and *Bacillus stearothermophilus* maltogenic amylase. The kinetics for the inhibition by the two analogues was studied for β-glucosidase, β-galactosidase, cyclomaltodextrin glucanosyltransferase (CGTase), and α-glucosidase. Both analogues were potent competitive inhibitors for β-glucosidase, with K_I values in the range of 0.04–2.44 μM, and the lactose analogues were good uncompetitive inhibitors for β-galactosidase, with K_I values in the range of 159–415 μM, while acarbose was not an inhibitor for either enzyme at 10 and 5 mM, respectively. Both analogues were also potent mixed inhibitors for CGTase, with K_I values in the range of 0.1–9.3 μM. The lactose analogue was a 6.4-fold better competitive inhibitor for α-glucosidase than was acarbose.

Keywords: Enzyme inhibitors; Acarbose; Isoacarbose; Cellobiose–acarbose analogues; Lactose–acarbose analogues; β-Glucosidase; β-Galactosidase; Cyclomaltodextrin glucanosyltransferase; α-Glucosidase; *Bacillus stearothermophilus* maltogenic amylase

1. Introduction

Acarbose is a pseudotetrasaccharide with an unsaturated cyclitol linked to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose, which is linked α-(1→4) to maltose (Fig. 1(A)). It is a well-known inhibitor of several carbohydrases, such as α-glucosidase,[1–3] dextransucrase,[4,5] alpha-amylase,[3,6,7] glucoamylase,[8] and cyclomaltodextrin glucanosyltransferase.[9,10] The nonreducing terminal, unsaturated cyclitol has been proposed to be an essential feature for the inhibition of the enzymes.[2]

Recently, we reported that *Bacillus stearothermophilus* maltogenic amylase (BSMA) was capable of cleaving the first α-(1→4) glycosidic linkage of acarbose and transferring acarviosine–glucose to various carbohydrate acceptors.[11] We found that when the acceptor was D-glucose, acarviosine–glucose was transferred to the C-6-OH group, giving isoacarbose (Fig. 1(B)), which was even a more potent inhibitor of α-glucosidase, alpha-amylase, and CGTase, with K_I values of 0.04–1.25 μM.[3] BSMA was also capable of transferring acarviosine–glucose from acarbose to the C-6-OH group of the nonreducing-end moiety of a variety of disaccharides, including cellobiose and lactose. We have pre-

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0008-6215/01/$ - see front matter
PII: S0008-6215(01)00016-7

pared and purified these cellobiose and lactose acceptor-products and have studied their inhibition of β-glucosidase [EC 3.2.1.21], β-galactosidase [3.2.1.23], CGTase [EC 2.4.1.19], and α-glucosidase [EC 3.2.1.20].

2. Experimental

Materials.—Acarbose was kindly supplied by Bayer Korea (Seoul, South Korea). Cellobiose and lactose, almond β-glucosidase (No. G4511), *Escherichia coli* β-galactosidase (No. G5635), and baker's yeast α-glucosidase (No. G5003) were purchased from Sigma Chemical Co. (St. Louis, MO). *Bacillus macerans* CGTase was prepared as previously reported.[3] BSMA was prepared as previously reported.[12] All other chemicals used were of reagent grade.

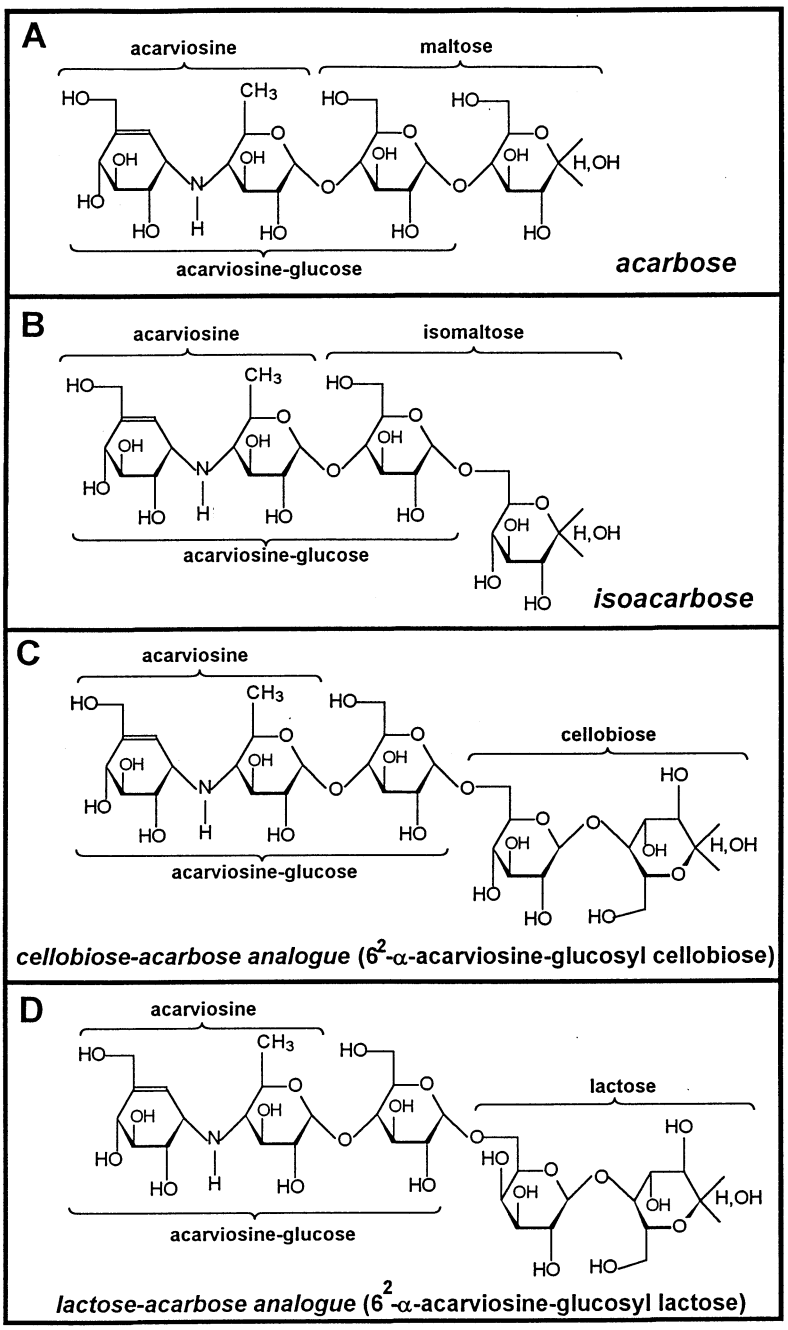

Fig. 1. Structures of acarbose and its analogues. A, acarbose; B, isoacarbose; C, cellobiose–acarbose analogue (6^2-α-acarviosine-D-glucosyl cellobiose); D, lactose–acarbose analogue (6^2-α-acarviosine-D-glucosyl lactose).

Preparation of cellobiose and lactose BSMA and acarbose acceptor-products.—The transglycosylation reaction of acarbose by BSMA was carried out in the presence of cellobiose and lactose according to a previous procedure.[11] BSMA was added to a mixture of 10% (w/v) of acarbose and 7% (w/v) of cellobiose or 10% (w/v) of lactose in 50 mM sodium citrate buffer (pH 6.0). The reaction was carried out for 72 h at 50 °C. The products were analyzed by TLC on Whatman K6F silica gel plates (Fisher Scientific, Chicago, IL) with 3:1:1 EtOAc–2-propanol–water. The carbohydrates were visualized on the TLC plate by dipping into 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine, 5% (v/v) H_2SO_4 in MeOH, followed by heating at 120 °C for 10 min.[13] Unreacted acarbose, cellobiose or lactose, acarviosine–glucose, and D-glucose were removed from the reaction mixture by gel-filtration chromatography on Bio-Gel P-2 (< 400 mesh) column (2 × 90 cm). The cellobiose or lactose acceptor-products were collected and further purified on TLC by several ascents (18.5 cm path length) with 3:1:1 volumes of EtOAc–2-propanol–water, until each compound was resolved. The separated products, corresponding to single spots on TLC, were removed from the silica gel plate and extracted with distilled water. The purity of each purified product was checked on TLC to give a single spot. This material was then used in the inhibition studies.

Enzyme reactions.—The reactions of β-glucosidase, β-galactosidase, and α-glucosidase were measured by determining the amount of D-glucose released from cellobiose, lactose, and maltose, respectively, using a micro glucose oxidase assay.[14] The β-glucosidase reaction was carried out in 50 mM acetate buffer (pH 5.0) at 37 °C. The β-galactosidase and α-glucosidase reactions were carried out in 50 mM phosphate buffer (pH 7.0) at 37 °C. The reactions were stopped by adding HCl to the digests to give a pH of 1. The reaction of CGTase was carried out using a maltodextrin (d.p. = 13), 50 mM imidazole–HCl (pH 6). The amount of cyclodextrins that formed was determined using Methyl Orange and measuring the absorption at 528 nm.[3,15] The kinetic parameters were calculated using software developed by Duggleby.[16]

Kinetics of enzyme inhibition.—The enzyme reactions for β-glucosidase, β-galactosidase, α-glucosidase, and CGTase were performed according to the foregoing reaction conditions with various concentrations of the inhibitors (concentrations of which were determined by quantitative TLC densitometry[13]). The type of inhibition was determined using Dixon plots and their replot of the slope versus the reciprocal of the substrate concentrations.[17] Inhibition constants were determined by using a non-linear regression analysis.[3]

3. Results

The transglycosylation reaction of cellobiose and lactose with acarbose and BSMA gave three products for each of the carbohydrate acceptors, compounds labeled **1**, **2**, and **3** in Fig. 2 for cellobiose and compounds **4**, **5**, and **6** in Fig. 2 for lactose. The major product for cellobiose was **3** and had been identified previously as 6^2-α-acarviosine-glucosyl cellobiose [6^2-α-agcel] and the two minor products (**1** and **2**) were 4^2- and 3^2-α-acarviosine-glucosyl cellobiose, respectively.[11] The major product for lactose was **5**, which was identified as 6^2-α-acarviosine-glucosyl lactose [6^2-α-aglac].[11] The structure of compound **5** in Fig. 2 was confirmed to be 6^2-α-acarviosine-glucosyl-lactose by ^{13}C NMR (data not shown). The two minor products (**4** and **6**) were 4^2- and 3^2-α-acarviosine-glucosyl-lactose.

The type of inhibition for each inhibitor was determined according to a Dixon plot and the replot of the slope. The inhibition constants, K_I and K'_I, were, respectively, the dissociation constants of the enzyme–inhibitor complex [EI] and the enzyme–substrate–inhibitor complex [ESI]. The relative potency of the inhibitors is given in Table 1, relative to the best inhibitor studied or to acarbose when it was an inhibitor.

Inhibition of almond β-glucosidase.—Almond β-glucosidase was inhibited by the cellobiose acceptor products, **1**, **2**, and **3**. All three compounds were shown to be competitive inhibitors from a Dixon plot for the inhibition of β-glucosidase. The inhibition constants (K_I) of **1**, **2**, and **3** were in the range of 0.11–0.81 μM (Table 1). Almond β-glucosi-

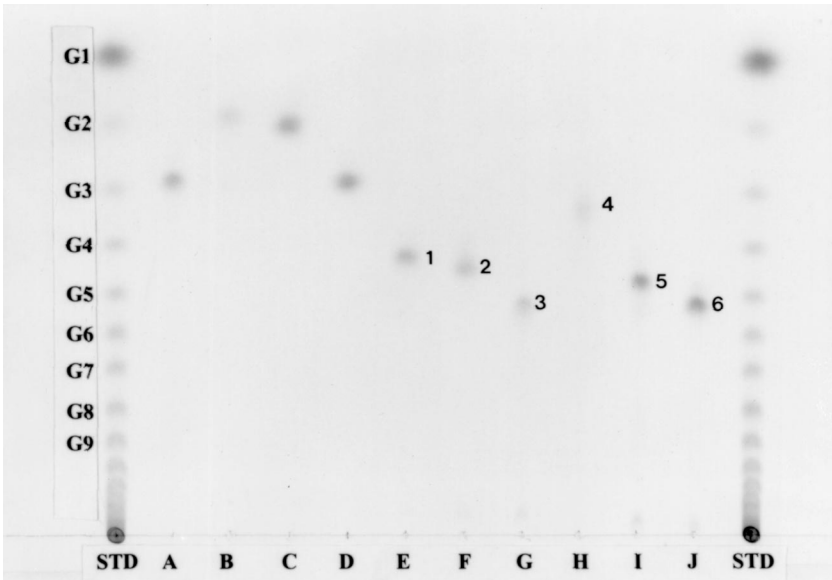

Fig. 2. Thin-layer chromatogram of the purified products of cellobiose and lactose acceptor reactions with acarbose by *Bacillus stearothermophilus* maltogenic amylase. STD, maltodextrin standards (G1–G9); lane A, acarbose; lane B, acarviosine–glucose; lane C, cellobiose; lane D, lactose; lane E, F, and G, cellobiose acceptor products of **1**, **2**, and **3**, respectively; lane H, I, and J, lactose acceptor products of **4**, **5**, and **6**, respectively.

Table 1
Inhibition constants of acarbose analogues containing cellobiose and lactose structures, for almond β-glucosidase, *E. coli* β-galactosidase, baker's yeast α-glucosidase, and *B. macerans* cyclomaltodextrin glucanosyltransferase (CGTase)

Enzyme	Inhibitors [a]	Type of inhibition	K_I (μM) [b]	K'_I (μM) [b]	Inhibitor potency [c]
β-Glucosidase	acarbose	no inhibition [>10 mM] [d]			
	1	competitive	0.81		4.9
	2 [6^2-α-agcel]	competitive	0.45		8.9
	3	competitive	0.11		36.4
	4	competitive	0.04		100.0
	5 [6^2-α-aglac]	competitive	0.52		7.7
	6	competitive	2.44		1.6
β-Galactosidase	acarbose	no inhibition [>5 mM] [d]		0.0	
	5 [6^2-α-aglac]	uncompetitive		159	100.0
	6	uncompetitive		415	38.3
	isoacarbose	competitive	430	37.0	
α-Glucosidase	acarbose	competitive	77.9		1.0
	5 [6^2-α-aglac]	competitive	12.3		6.3
CGTase	acarbose	mixed	2.5	3.1	1.0
	1	mixed	2.3	6.7	1.1
	2	mixed	0.8	7.2	3.2
	3	mixed	0.3	9.3	8.7
	4	mixed	0.1	5.4	21.1
	5	mixed	1.2	2.2	2.2
	6	mixed	1.5	3.7	1.6

[a] Inhibitors, cellobiose and lactose acceptor products, are designated as numbers from the TLC (Fig. 1).

[b] K_I is the inhibition constant, defined as [E][I]/[EI]; K'_I is the inhibition constant, defined as [ES][I]/[ESI].[17]

[c] Inhibitor potency for β-glucosidase and β-galactosidase are given based on assigning the best inhibitor a value of 100 and dividing the K_I of the best inhibitor by the K_I of the inhibitor, multiplied by 100; the inhibitor potency for α-glucosidase and CGTase is based on how many times better the inhibitor was than acarbose.

[d] Acarbose showed no inhibition for β-glucosidase and β-galactosidase up to 10 and 5 mM, respectively.

dase was also competitively inhibited by the lactose acceptor products of **4**, **5**, and **6**, with K_I values in the range of 0.04–2.44 μM. Inhibitors **4** and **5** were 68- and five-times, respectively, stronger inhibitors than was **6**. Among the six inhibitors, **4** was the best inhibitor for β-glucosidase, with a K_I of 40 nM. In contrast, acarbose showed no inhibition for almond β-glucosidase, even at a concentration of 10 mM.

Inhibition of E. coli *β-galactosidase.*—Inhibitors **5** and **6** inhibited *E. coli* β-galactosidase, uncompetitively, with K_I' values of 159 and 415 μM (Table 1); **5** was approximately three-times as effective as **6**. On the other hand, isoacarbose showed a competitive inhibition, with K_I of 430 μM. Acarbose showed no inhibition for β-galactosidase up to 5 mM. Due to the relatively high inhibition constants for β-galactosidase and the limitation of the amounts of material, inhibitors **1**–**4** for the inhibition of β-galactosidase were not studied.

Inhibition of B. macerans *CGTase.*—The inhibition of *B. macerans* CGTase by the six inhibitors, **1**–**6**, was a mixed type of noncompetitive inhibition with K_I values in the range of 0.1–2.3 μM. By comparing the K_I values for acarbose,[3] all six inhibitors, **1**–**6**, were found to be equally or much more effective inhibitors for CGTase than acarbose in the range of 1–21-times.

Inhibition of baker's yeast α-glucosidase.—Compound **5** [6^2-α-aglac] inhibited baker's yeast α-glucosidase competitively with K_I of 12.3 μM. By comparing the K_I values, it was found that **5** was six-times more effective inhibitor than acarbose (Table 1).

4. Discussion

Many, if not most, K_m values for carbohydrases are in the mM range, as are most K_I values for substrate analogue inhibitors.[18] The K_I values for the α-acarviosine–glucose-substituted cellobiose and lactose compounds reported here, however, are in the μM and even some in the nM range for β-glucosidase and β-galactosidase, indicating that they are very effective inhibitors. This increase in one to two orders of magnitude inhibitory potency must be attributed to the cellobiose and lactose structures that are combined with acarviosine–glucose structure. Acarviosine contains the unsaturated cyclitol unit at the nonreducing end of the inhibitors, and the unsaturated cyclitol has been postulated to act as a transition-state analogue of the intermediate that is formed in the cleavage of the glycosidic linkage during enzymatically catalyzed hydrolysis.[2] We, however, further postulate that the potency of the inhibition is also due to the nitrogen–glycose linkage of the cyclitol unit to 4-amino-4-deoxy-D-quinovose. Under the pH conditions, optimum for the enzymes, this nitrogen–glycose linkage is protonated to give a positively charged nitrogen atom. This positive charge on the glycosidic nitrogen mimics even more the transition-state for cleaving a glycosidic linkage than does the unsaturated cyclitol. The positive charge mimics the positive charge that is incipient on carbon during the cleavage of the glycosidic linkage and the protonated nitrogen further mimics the protonation of the leaving glycosidic oxygen.

X-ray diffraction analysis of crystals of β-glucosidases, β-galactosidases, and glucoamylase show that their active sites are pockets in which the nonreducing ends of the substrates enter the pockets first. Acarbose has α-acarviosine attached to C-4 of maltose. Acarbose is a potent inhibitor of glucoamylase.[8] The acarbose analogues in this study have acarviosine-α-D-glucose attached to the C-6 positions of the nonreducing ends of cellobiose and lactose. The acarviosine group of these inhibitors must also fit into the active site pockets of β-glucosidase and β-galactosidase much as acarbose fits into the active site pocket of glucoamylase. But, because acarbose was not an inhibitor for these enzymes, it is concluded that the presence of the β-(1→4) glucosidic linkage and the β-(1→4) galactosidic linkage in the cellobiose and lactose analogues, respectively, make them specific active site directed inhibitors for the β-glycosidases.

Specific enzyme inhibitors of high potency are of potential interest in food, pharmaceutical, medical, and agricultural applications. The preparation of acarviosine–glucose analogues of acarbose by the transglycosylation reactions of BSMA with acarbose and carbo-

hydrate acceptors gives a new class of versatile inhibitors that have high inhibitory potency and active-site-directed specificity that is dependent on the structure of the acceptor.

In conclusion, we have shown that the transfer of an acarviosine–glucosyl group from acarbose to cellobiose and lactose by the action of BSMA gives highly effective and specific inhibitors for β-glucosidase, β-galactosidase, and some other related amylolytic enzymes by having the cellobiose and lactose structures present in the acarbose molecule, thereby providing active site directed inhibitors.

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