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Note

Action of human alpha amylases on reduced malto-oligosaccharides

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Kinetic studies on the reactions of human-pancreatic and -salivary alpha amylases with a series of reduced malto-oligosaccharides (maltotetraoitol through maltoheptaoitol) were conducted. Maltotetraoitol was barely hydrolyzed by either alpha amylase under the conditions used, and the minimum size of substrate susceptible to hydrolysis was maltopentaoitol. Among these substrates, maltohexaoitol showed the highest reactivity with each alpha amylase, especially with salivary alpha amylase, compared with maltopentaoitol or maltoheptaoitol. For the combination of each enzyme with each substrate, the predominant point of cleavage of the substrates was found to be the third (1→4)-α-D-glucosidic linkage from the D-glucitol residue.

INTRODUCTION

Robyt and French[1] found that porcine-pancreatic alpha amylase [(1→4)-α-D-glucan 4-glucanohydrolase (EC 3.2.1.1)] preferentially splits the second (1→4)-α-D-glucosidic linkage from the (reducing) D-glucose residue of a series of malto-oligosaccharides** (maltotetraose, G_4, through maltoheptaose, G_7). The same result was obtained with human-pancreatic and -salivary alpha amylases[2]. In the meanwhile, Parrish *et al.*[3] had reported that modification at C-1 of the (reducing) D-glucose residue of the malto-oligosaccharides does not cause any change in the enzymic behavior of starch-metabolizing enzymes, including human-salivary alpha amylase (HSA), but they did not mention the effect of the modification on the action patterns of these enzymes.

In contrast, when the reduced malto-oligosaccharides were compared with the

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**Abbreviations: G_n designates a pure, individual, linear malto-oligosaccharide composed of n D-glucose units; R_n, a reduced malto-oligosaccharide modified at C-1 of the (reducing) D-glucose residue of G_n; HSA, human-salivary alpha amylase; HPA, human-pancreatic alpha amylase.

unreduced substrates in terms of the action patterns of human alpha amylases, by kinetically analyzing the amounts of products, we obtained a result somewhat different from that of Parrish *et al.*[3]. We now describe the effects of modification of the substrate on the action patterns of human alpha amylases, and also a shift of a major cleavage point in these substrates.

MATERIALS AND METHODS

Enzymes. — Human-parotid saliva (50 mL) was collected preprandially from several adults, and was used for purification of salivary alpha amylase (HSA) according to the method of Fischer and Stein[4]. Human-pancreatic alpha amylase (HPA) was purified by the method of Stiefel and Keller[5]. *Rhizopus* glucoamylase (EC 3.2.1.3) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Purified *Bacillus licheniformis* alpha amylase was prepared as previously described[6].

Preparation of reduced malto-oligosaccharides. — Each malto-oligosaccharide was prepared from a partial hydrolyzate of amylose with *Bacillus licheniformis* alpha amylase, and purified as previously described[6].

Reduction of maltodextrin was achieved with sodium borohydride solution[7]. After incubation, with stirring, for 20 h at room temperature, the mixture was acidified with dilute acetic acid, to decompose the excess of sodium borohydride, treated with Dowex 50W (H^+) resin, and the suspension filtered. The filtrate was evaporated to dryness *in vacuo*, methanol was added to the dry residue, and the mixture was re-evaporated, the last procedure being repeated once. The residue was dissolved in distilled water, the solution was treated with charcoal, the suspension filtered (Millipore filter, 0.45 μm), and the filtrate evaporated to dryness *in vacuo*.

Enzyme reaction. — The substrates, namely, reduced malto-oligosaccharides, were prepared in aqueous solution at concentrations of 2.5–25 mg per mL. For determination of the rates of reactions, the reaction mixture initially contained 0.05M phosphate buffer, pH 7.0 (0.4 mL), and human alpha amylase (3 units in 0.05M phosphate buffer, pH 7.0, containing 40mM NaCl and 5mM $CaCl_2$) (0.4 mL) in a total volume of 1.0 mL. After incubation for 10 min at 37°, the reaction was stopped by adding M acetic acid (0.2 mL), and the mixture was submitted to quantitative estimation of reduced and unreduced malto-oligosaccharides by paper chromatography. The initial rate of the reaction was determined as a function of the substrate changed.

Quantitative paper-chromatography. — Toyo No. 50 filter paper, to which an appropriate amount of the reaction mixture was applied, was irrigated three times at 30° in the descending mode with 7:3 (v/v) 1-propanol–water. To check the position of each carbohydrate on the paper, controls of the chromatogram, both treated and untreated with glucoamylase, were monitored by the silver nitrate dip-method[8]. Each section of carbohydrate on the chromatogram was excised, and then extracted with distilled water in a test tube in a boiling-water bath for 15 min, cooled, and filtered; the carbohydrate content of the filtrate was determined by the anthrone method[9].

RESULTS AND DISCUSSION

Susceptibility to hydrolysis. — In contrast to the result obtained in the previous study, that both of the human alpha amylases hydrolyze maltotetraose (G_4) at a significant rate[2], maltotetraoitol (R_4) was found to be resistant to both enzymes under the same conditions. Maltopentaoitol (R_5) was degraded by both enzymes, but the rate of hydrolysis decreased in comparison with that of G_5; namely, at the initial concentration of substrate (4 mg per mL of reaction mixture), the rate of hydrolysis of R_5 was approximately half to one-third that of G_5. On the other hand, maltohexaoitol (R_6) was much more rapidly degraded by both enzymes than maltohexaose (G_6), especially by HSA, and, at the initial substrate-concentration of 4 mg per mL, the rate increased approximately 4- to 5-fold with HSA, compared with that for its unmodified form, G_6. Maltoheptaoitol (R_7) was also a better substrate than maltoheptaose (G_7) and showed an increase of 20–140% in the rate of hydrolysis compared to maltoheptaose (G_7).

TABLE I

HYDROLYSIS OF REDUCED MALTO-OLIGOSACCHARIDES, AND FORMATION OF PRODUCTS BY HUMAN ALPHA AMYLASES[a]

Alpha amylase	*Substrate added*	*Carbohydrates in hydrolyzate (μg/mL)*	*Initial substrate-concentration (mg/mL)* 1.0	2.0	3.0	4.0	6.0	8.0	10.0
HPA	R_5	G_2 formed	14	25	38	51	65	77	88
		R_3 formed	20	37	56	76	99	113	130
		R_5 residual	967	1939	2908	3876	5842	7819	9786
	R_6	G_3 formed	32	60	90	134	168	208	241
		R_3 formed	32	60	90	134	169	208	241
		R_6 residual	937	1883	2824	3737	5669	7591	9527
	R_7	G_2 formed	1	3	4	6	8	10	11
		R_3 formed	16	30	43	56	75	97	112
		G_4 formed	19	37	55	71	99	121	141
		R_7 residual	964	1933	2900	3870	5819	7776	9741
HSA	R_5	G_2 formed	10	18	28	36	48	58	64
		R_3 formed	14	26	41	52	72	85	95
		R_5 residual	977	1957	2933	3914	5883	7861	9844
	R_6	G_2 formed	6	13	18	21	30	38	42
		G_3 formed	255	468	700	880	1249	1513	1727
		R_3 formed	256	468	703	882	1252	1517	1730
		R_4 formed	12	22	34	42	59	72	82
		R_6 residual	479	1000	1567	2195	3451	4912	6475
	R_7	G_2 formed	8	16	24	31	43	53	59
		R_3 formed	38	71	105	140	196	239	269
		G_4 formed	42	84	120	160	222	272	306
		R_7 residual	913	1835	2756	3674	5546	7447	9378

[a]Experimental conditions are indicated in the text.

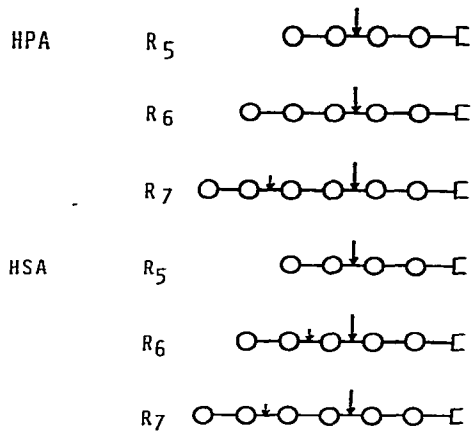

Fig. 1. The distribution of the cleavage points during initial action of both human alpha amylases on reduced malto-oligosaccharides. [Key: ○, a non-reducing D-glucosyl unit; □, a D-glucitol unit; —, a (1→4)-α-D-glucosidic bond; ↓, a major cleavage point; and ↓, a minor cleavage point]

It is noteworthy that R_6 showed remarkable reactivity with HSA, corresponding to nearly 8 times that with HPA. Among unmodified malto-oligosaccharides, maltotriaose (G_3) was almost immune to both alpha amylases, and the minimum size of substrate to be readily hydrolyzed was G_4, having three (1→4)-α-D-glucosidic linkages, as described previously[2].

However, modification of the (reducing) D-glucose residue of the malto-oligosaccharides to a D-glucitol residue required R_5 as the limiting substrate for the enzymes. Furthermore, quite similar results were obtained with such oxidized derivatives of malto-oligosaccharides as maltotrionic, maltotetraonic, maltopentaonic, maltohexaonic, or maltoheptaonic acids (unpublished data).

Action pattern. — Table I gives the quantitative distribution of the products when the reduced derivatives were acted on by HPA and HSA. No substantial difference was found between HPA and HSA in the early stage of hydrolysis, *i.e.*, R_5 was cleaved into R_3 and G_2 in a mode of single attack; R_6 mainly into G_3 and R_3; and R_7 was multi-attacked, to produce R_3, G_4, and a trace of G_2. The only actions of HSA different from those of HPA were the ability to form small amounts of G_2 and R_4 from R_6, and the extreme reactivity of HSA in hydrolyzing R_6. With both enzymes, at least part of the G_2 formed from R_7 might be attributed to G_4, originating from R_7. In any combination of each enzyme and each reduced malto-oligosaccharide, maltitol (R_2) was never produced. On the basis of the data in Table I, the cleavage patterns of human alpha amylases for R_5, R_6, and R_7 are shown qualitatively in Fig. 1.

Thus, it may be assumed that a major bond cleaved in the reduced malto-oligosaccharide, which was reduced at C-1 of the (reducing) D-glucose residue of the naturally occurring oligosaccharide, is the third (1→4)-α-D-glucosidic linkage from the D-glucitol residue. Maltotetraoitol was, however, barely attacked by either

TABLE II

K_m AND V_{max} VALUES OF HUMAN ALPHA AMYLASES FOR REDUCED MALTO-OLIGOSACCHARIDES

Alpha amylase	*Substrate*	K_m		V_{max} (ΔRn, *mg/mL*)	V_i[a] *(mg/mL)*
		(mg/mL)	*(mM)*		
HPA	R_5	15 2	18.3	0.58	0.124
	R_6	19.2	19.4	1 33	0.263
	R_7	23.3	20.1	0.98	0.131
HSA	R_5	14.1	17.0	0 37	0.086
	R_6	23.8	24.0	11.1	1.805
	R_7	27.0	23.4	2.50	0.326

[a]Rate of reaction (V_i) expressed as the amount of substrate changed at the initial substrate-concentration of 4 mg/mL.

alpha amylase under the conditions described, although it also has three (1→4)-α-D-glucosidic linkages.

Kinetic parameters. — Table II shows the kinetic parameters for reduced malto-oligosaccharides (R_5, R_6, and R_7) with both alpha amylases. In this Table, V_i expresses the rate of reaction at the initial substrate concentration of 4 mg per mL. The K_m values for the reduced malto-oligosaccharides lay in the range 17.0–24mM, but these values were ~10 times those for unmodified malto-oligosaccharides. The value of V_{max} varied, depending on the size of the substrate. Among these, the value of V_{max} for HSA with R_6 was remarkably larger than the values for the other substrates, suggesting high reactivity of R_6 with HSA, despite its rather large K_m value.

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