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ORIGINAL ARTICLE

The digestable parent cyclodextrin

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Abstract The enzymatic digestibility of parent γ-cyclodextrin by human saliva α-amylase was investigated aiming at the determination of lifespan of intact macro ring. It was found that the ring-opening reaction was the slowest step of the γ-cyclodextrin degradation process. The reaction products were mainly maltose and malto-triose, while no higher malto-oligomers were detected. The enzymatic degradation of γ-cyclodextrin reduces the possibility of influencing bioavailability of nutritional lipophiles or drug actives co-administered with γ-cyclodextrin as an excipient or additive. Though there are numerous papers on the ring opening of cyclodextrins by amylases and the same capability of the human α-amylase is expected now we prove this activity. The hydrolysis reaction was followed by direct measurement of the resulting maltose and maltotriose, for the first time.

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Introduction

Cyclodextrins (CDs) and their derivatives are used as carriers of many pharmaceutical derivatives. Although the number of the glucose units forming the cyclodextrin ring can be more than 8, practically the α-, β-, and γ-cyclodextrins having 6, 7, and 8 sugar units, respectively, are used in the industrial applications [1, 2]. When the guest compounds leave the cyclodextrin ring, the empty apolar cavity of cyclodextrin may entrap other suitable molecules around and this may cause alterations of the bioavailability and/or ADME (absorption, distribution, metabolism, and excretion of pharmaceutical compounds in the organism) properties of biologically active lipophiles occurring in the living organism. Parent cyclodextrins themselves occur in certain starch-based processed foods such as bread, corn syrups and beer, and by this way CDs have been consumed in small quantities by humans for a long time [3]. While the α- and β-cyclodextrin rings are rather rigid, the γ-cyclodextrin torus has some flexibility and thus is expected to be a more attractive substrate of glycosidase enzymes, such as the human amylases.

Earlier studies by Marshall and co-workers indicated that γ-cyclodextrin was hydrolyzed by human salivary and pancreatic α-amylase at appreciable rates [4]. The optimum pH for these enzyme reactions at 37 °C in the presence of 0.1 M NaCl was at around pH 5, which was different from the optimum pH (pH 6.9) of the enzymes for starch. The hydrolysis of γ-CD by both enzymes took place via the so-called multiple attack mechanism. The kinetic study was based on the reductive property of the glucose which is

the hydrolysis product of the malto-oligomers by glucoamylase added to the reaction mixture. The opening of the γ-cyclodextrin ring results in a linear malto-octaose which is therefore a non-complex forming oligosaccharide and it can be a further substrate of the amylase enzymes.

In foods, γ-CD may be used as a carrier for flavors, vitamins, polyunsaturated fatty acids, and other ingredients. It also has useful properties as a flavor protectant or stabilizer in different combined food systems [5]. The daily intake from all its intended uses in food at highest feasible concentrations has been established at 4.1 g/person/day for consumers of γ-CD containing foods [6].

All the toxicology safety studies demonstrated that γ-CD is well tolerated and elicits no toxicological effects. Metabolic studies in rats showed that γ-CD is rapidly and essentially completely digested by salivary and pancreatic amylase [7]. Therefore, the metabolism of γ-CD closely resembles that of native starch and linear dextrins. A human study with ingestion of single doses of 8 g of either γ-CD or maltodextrin did not reveal a difference in gastrointestinal tolerance of these two products [8].

The active center of amylase enzymes has several subsites and the reaction speed depends on the length of the malto-oligomer [9, 10]. This reaction was studied in details by Kandra et al. [11–13] and a computer program was developed to determine the binding energy of the subsites of active center [14]. The number of subsites may vary depending on the amylase type but the number of the subsites at the reducing end from the cleavage point (aglycon site) is never less than two [15]. Maltose itself is not a substrate of the amylase enzymes, which means that more than two subsites are necessary to hydrolyze the glycosyl bond. The geometry of the active center of α-amylase enzymes adapt to the shape of the substrates. Maltodextrins have a helical conformation which is distorted into a more linear conformation by the amylase enzyme during the formation of enzyme–substrate complex. The α- and β-cyclodextrin rings have lack of conformation flexibility therefore they are very slowly hydrolyzed by amylase enzymes. The hydrolysis of α-, β- and γ-cyclodextrins by *Aspergillus oryzae* α-amylase was studied at pH 5.2 and 37 °C. The kinetic parameters were determined and while the V_{max} value increased markedly in the order α-, β- and γ-cyclodextrin the K_m values did not show significant difference [9]. Some flexibility of γ-cyclodextrin ring makes it susceptible for opening by amylases. The number of the sugar units to form a substrate-enzyme complex in case of cyclodextrins is limited therefore the first step in the digestion of cyclodextrin, the ring opening, is the slowest step.

Some biochemical effect like glycemic effect of ingested γ-cyclodextrin was investigated by Asp et al. [16] and they found that the postprandial glycemic and insulinemic response was attenuated by γ-cyclodextrin consumption compared to the effect of maltodextrin consumption. They concluded that the γ-cyclodextrin was slowly but completely digested consequently the intake of γ-cyclodextrin can be regarded a slow-release source of glucose.

For understanding the enzymatic cyclodextrin hydrolysis Bruedenberger et al. [17] prepared and examined several bacterial enzyme-cyclodextrin complexes in crystal forms. They found that the active centers of all enzymes are similar and they are located at the same place of the protein chain. The binding of the cyclodextrin rings are at the subsites (−2), (−1) and (+1) in all cases and the cyclodextrin rings are deformed mainly by the binding of 6-hydroxyl group at the (+1) subsite.

Recently a very detailed study was published by Mótyan et al. [18] providing the binding energy of subsites of α-amylase enzymes. It is shown that the α-amylase enzymes have at least 3 binding subsites at the glycone site and it has also minimum 3 binding subsites at the aglycone site.

In this work, the human amylase was used for the digestion analyses of γ-cyclodextrin proving that γ-cyclodextrin could be opened and digested within short time after administration and γ-cyclodextrin has no effect on other biochemical processes. Although we found earlier that several food or beverages consist of glycosylated cyclodextrins [3] the fate of consumed γ-CD in humans has not been investigated. The enzymatic reactions were followed by HPLC analysis using evaporative light scattering detector detecting directly the reaction products of α-amylase enzyme.

Experimental

Enzyme

α-Amylase (EC 3.2.1.1) from human saliva (Type IXA Sigma, specific activity: 1,000–3,000 units/mg protein) was used for the enzymatic reaction. This enzyme gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and possessed no α- and β-glycosidase activity. 0.167 mg of α-amylase was dissolved in 1.0 mL distilled water.

Substrate

γ-Cyclodextrin (Wacker Chemie, Munich, Germany, no detectable impurity by HPLC) 10 mg was dissolved in 1 mL pH 6.7 phosphate buffer.

Malto-oligomer standards

The malto-oligomer standards were obtained by enzymatic degradation of dextrans followed by column chromatography and lyophilization [19]. The structures were proved

by ^{13}C-NMR measurements. No detectable impurities by HPLC.

Enzymatic reactions

The reactions were performed in HPLC autosampler vials. The vials were thermostatted at 40 °C. The reaction was followed by HPLC for 64 h. The enzyme solution (0.05 mL) was added to substrate solution (1.0 mL). The sampling was made from the reaction mixture directly.

HPLC conditions

The HPLC analyses were carried out on a HP 1050 series system including quaternary gradient pump, automatic sampler, SandraSelerity 9000 series column oven thermostatted at 40 °C and equipped with solvent preheater. An Alltech ELSD 2000 type evaporative light scattering detector was used for detecting the free sugar compounds. The detector was calibrated and the linearity of the response in the expected concentration range was checked using free malto-oligomers. The HPLC analyses were performed on a Phenomenex Luna column (5μ NH_2 100A 250 mm × 4.6 mm).

Eluent

Acetonirile–water 35:65 vol/vol, isocratic.

Data acquisition and evaluation

Agilent ChemStation A.10 version software was used to control the HPLC system and to acquire the data from the detector via A/D converter. A Gerstel made add-on software module controlled the column oven.

Results and discussion

The enzymatic digestion reaction followed by HPLC resulted in mainly maltose and lower amounts of malto-triose (Fig. 1). Although there were higher malto-oligomer standards available, those digestion products were not detectable at any part of the digestion reaction. Glucose could be detected only at the very end of the reaction in small quantity (see Fig. 1).

The results of HPLC analysis showing the change of the concentrations of γ-CD and malto-oligomers during the enzymatic degradation are presented in Table 1. The identification of the components are based on retention time, the concentration of them is calculated using the peak area and calibration data. The peaks of maltose and malto-triose are partially overlapping what may cause some quantitation error. The peak resolution is R = 1 the theoretical relative error is less then 5%.

Based on these results the proposed reaction pathway is shown in Fig. 2. The slowest step of the enzymatic reaction is the ring opening. The result of the ring opening is malto-octaose, which is a good substrate of amylase enzymes. The preferred hydrolytic path is the removal of maltose units, resulting successively in malto-hexaose, malto-tetraose, and maltose. The hydrolysis of malto-hexaose to two malto-triose molecules is also possible. The less favorable path is the cutting malto-triose from malto-octaose resulting a malto-pentaose which is hydrolyzed to an additional

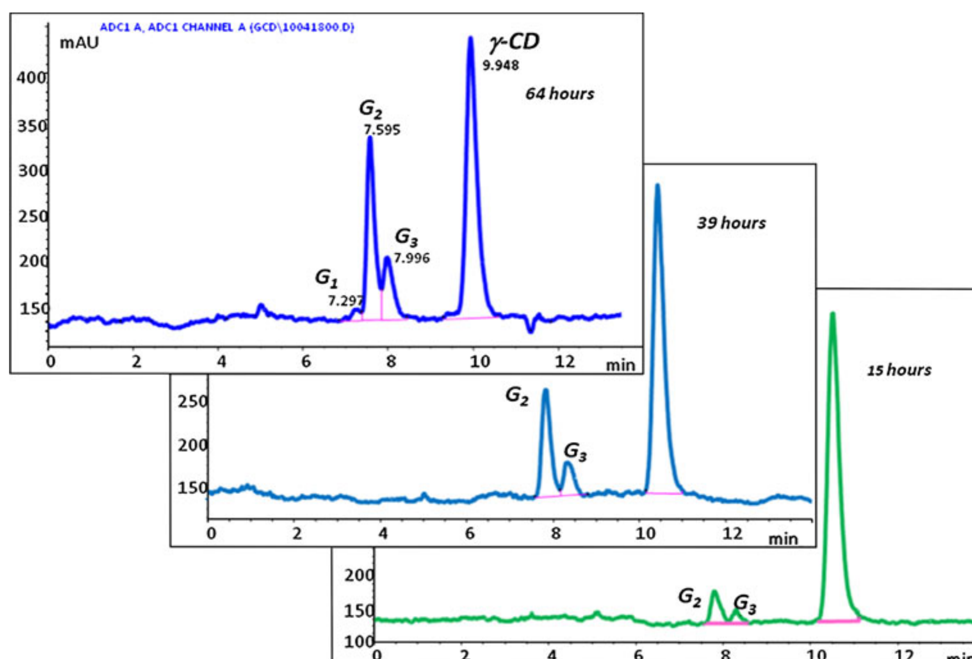

Fig. 1 HPLC chromatograms of the enzymatic reaction mixture (15, 39, and 64 h reaction time). The signals from the detector are digitalized by analogue to digital converter, and the intensity corresponds to the UV detector in mAU units shown on the scale

Table 1 HPLC analysis results as the function of the reaction time

Reaction time (h)	GCD (%)	G3 (%)	G2 (%)	G1 (%)
0:47	100.00	0.00	0.00	0.00
15:18	85.62	5.21	9.18	0.00
24:28	81.55	4.97	13.48	0.00
39:51	72.15	7.21	20.63	0.00
63:57	58.85	11.57	27.95	1.62

malto-triose and one maltose. Theoretically it is also possible, that the α-amylase enzyme cuts malto-octaose to two malto-tetraose first and then the two malto-tetraoses are hydrolyzed to maltose. Therefore the quantitative data of HPLC analysis may give only a rough estimation on the ratio of the possible directions as the products of the enzymatic ring opening reaction are substrates of α-amylase enzyme. The preferred path seems to be the cleavage of maltose unit from malto-octaose as it is shown in Fig. 2.

Figure 3 shows graphically the analysis results as a function of the reaction time. The ratio of maltose and malto-triose is almost the same during the reaction suggesting that the mechanism is a mixture of the mechanisms shown in Fig. 2. As the enzymatic reaction is not a simple one-substrate reaction the fitting curve data are only for calculation with no meaning on the reaction kinetics.

In our work we proved that the human saliva α-amylase enzyme is able to open the γ-CD ring and immediately after the ring opening the hydrolysis of the malto-octaose performs very quickly. This enzymatic reaction is very similar to other α-amylases activities as the amylase enzymes show high similarities in amino acid sequence and conformation. But still the experimental prove was missing for human α-amylases.

Conclusion

Our results contribute to understanding the mechanism of enzymatic digestion of γ-CD. The active center of amylase enzymes has several subsites and the reaction rate depends on the length of the malto-oligomer [2]. Although the shape of the cyclodextrin ring is similar to the helical shape of starch and glycogen, the enzyme distorts it to almost straight form and the glucose units are bound by the subsites of the enzyme. While only two glucose units are available for the enzyme–substrate formation in the case of α- and β-cyclodextrins they cannot be opened by the human α-amylases. However, the γ-cyclodextrin ring has certain flexibility and the enzyme can bind three glucose units (and not more), so that the enzyme is capable of opening the γ-CD ring. Since the possible number of the glucose units to form an enzyme–substrate complex with γ-CD is limited, the first step in the digestion process of γ-cyclodextrin, namely the ring opening, will be the slowest step, though still occurs.

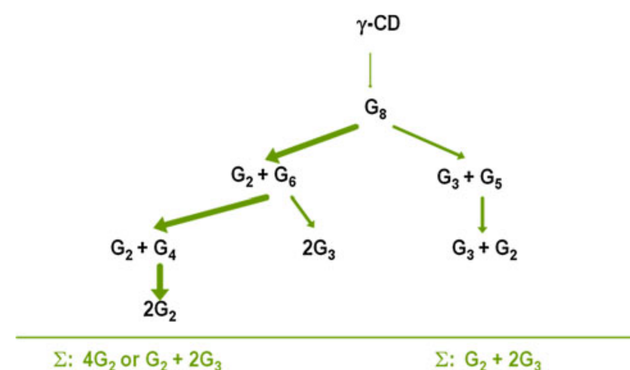

Fig. 2 Reaction pathway according to the products detected from the reaction mixture. (*G8* malto-octaose, *G6* malto-hexaose, *G5* malto-pentaose, *G4* malto-tetraose, *G3* malto-triose, *G2* maltose)

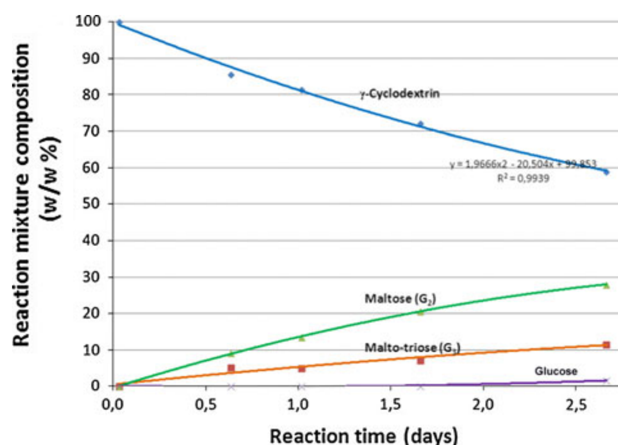

Fig. 3 Composition of the reaction mixture during the reaction time converted to days (see Table 1). (The regression equation of the GCD concentration is not related to the reaction kinetics)

It is supposed that the pancreas enzymes act similarly to the saliva amylases, and the digestion of cyclodextrin is further continued within the human gastrointestinal digestion system. This enzymatic reaction is similar to the other amylase-catalyzed enzymatic reactions [3].

The fact that γ-CD is easy to digest helps to dispel the concerns on its biochemical effects when applied in drugs and foods.

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