# ENZYMATIC DEGRADATION OF CYCLODEXTRINS; PREPARATION AND APPLICATION OF THEIR FRAGMENTS

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During the enzymatic degradation of γ-cyclodextrin by Aspergillus oryzae α-amylase all of the maltooligomers from maltose to maltooctaose were detectable in the reaction mixture. We have succeeded in the isolation of every single maltooligomer in pure form. The qualitative and quantitative distribution of the maltooligomers were determined by HPLC during the enzymatic hydrolysis. We used the maltooligomers in the enzyme kinetic investigations as both substrates and inhibitors. Kinetic parameters were determined and compared in each case. The application of these maltooligomers in preparative carbohydrate chemistry is in progress. We can use them as the carbohydrate components of artificial antigens. Other derivatives of maltooligomers, having diagnostic and enzymanalytical importance, have also been prepared.

## Introduction

Recently many studies have been made in connection with the interaction between carbohydrate degrading enzymes and cyclodextrins (1-9).

Hanrahan et al. (9) have studied the degrading ability of Takaamylase A on α- and β-cyclodextrins and these investigations have been extended to γ-cyclodextrin, as well, by Suetsugu et al. (10). It was shown that the enzymatic hydrolysis of γ-cyclodextrin led mainly to the formation of maltotriose; a small amount of maltotetraose was also present in the hydrolysate of both β- and γ-cyclodextrins but no higher-membered oligomers could be detected.

The substrate specifity of Bacillus subtilis amylase was studied by Robyt and French (11) by using maltodextrins as well. These authors employed, among others, maltooctaose as a substrate for the investigation of Bacillus polymixa

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amylase (12). Abdullah et al. (6) applied cyclic and linear $G_8$ as the substrate when examining the mechanism of action of α-amylases.

In previous studies (13) we have investigated the enzymatic degradation of α-, β- and γ-cyclodextrins with Aspergillus oryzae α-amylase and comparison has been made between the time-change of the kinetic parameters and the data of liquid chromatographic measurements providing information about the qualitative and quantitative composition of the mixture produced upon enzymatic reaction. It was supposed that the higher-membered maltooligomers formed in larger quantities in the first period of the reaction of γ-cyclodextrin could be competitive inhibitors of the enzymatic reaction, whereas the lower-membered maltooligomers, enriched in the subsequent stage of the process, could be non-competitive inhibitors.

The present work is aimed at the confirmation of the above assumption by means of the application of the maltooligomers – prepared in pure form – for the determination of the substrate-specifity of Aspergillus oryzae α-amylase, as well as for inhibition studies on the enzymatic hydrolysis of γ-cyclodextrin.

The application of these maltooligomers in preparative carbohydrate chemistry is in progress.

## Materials and methods

γ-cyclodextrin is a product of the Chinoin Pharmaceutical Works (Budapest) and its purity was min 99% after drying to constant weight. No other carbohydrate component could be detected by t.l.c. or h.p.l.c.

### α-amylase

The crystalline Aspergillus oryzae enzyme was obtained by a multistep purification produce (BIOGAL Pharmaceutical Works, Debrecen). The enzyme was electrophoretically homogeneous and in anhydrous state it could be stored with unchanged activity for several years. The $p_H$ optimum in phosphate buffer is 4.8-6.6. Its critical inactivation temperature is 65°C and the optimal temperature 35-37°C. Specific activity: 900-1000 SKE/mg protein N. The enzyme preparation contained a few percent of polysaccharide.

### Methods

The enzyme reactions were carried out in 0.2 M acetate buffer at $p_H$ 5.2 and 37°C by adding the enzyme solution to the substrate solution in a test tube with a micropipette. At appropriate intervals 0.2 ml samples of the hydrolysate were taken and the reducing values were measured by the Somogyi-Nelson method (14).

Simultaneously, the hydrolysate were analyzed by liquid chromatography. These studies were accomplished with a Hewlett-Packard 1081 liquid chromatograph by using a 250 mm long column of 4.6 mm internal diameter and 10 μm $NH_2$-type package. The chromatograms were visualized with a Linseis 2000 recorder, the curves were digitalized with a HP-9825 A computer. For identification of the components solutions of pure glucose and maltose were used. The additional components were identified on the basis of their retention times and according to the elution principles. For the preparation of the maltooligomers the enzymatic hydrolysate was subjected to heat-denaturation at an appropriate time, the supernatant was freeze-dried after centrifugation and acylated with acetic anhydride in pyridine by stirring for 24 h at room temperature and then for 1 h at 60°C. After working up the syrupy mixture of the maltooligomer-acetates was separated by short column chromatography (15), by using Kieselgel G (30 g/1 g sample) and 1:1 dichloromethane-ethyl-acetate as the eluent. The fractions were investigated by a thin layer chromatographic method applying pentaacetyl-D-glucose as the standard material. The unsubstituted maltooligomers were readily prepared by means of Zemplén deacetylation (16). For t.l.c. examinations a 3.5:5:1.5 butanol-acetone-water mixture was used. For developing solution a 5:5:1 mixture of 2% diphenylamine in acetone, 2% aniline in acetone and 85% $H_3PO_4$ was applied. Visualization of the chromatograms was accomplished by heating for 2 min at 120°C.

## Results and discussion

The enzymatic hydrolysis of γ-cyclodextrin with Aspergillus oryzae α-amylase was studied by monitoring of the composition of the reaction mixture and by identifying the hydrolysis products with h.p.l.c. method. The product distribution of the enzymatic hydrolysis of γ-cyclodextrin after 20, 30, 60 and 90 min reaction time is given in Table I. (In this case maltose could not be separated from the additional components.)

Appropriate work-up of the hydrolysate, involving purification, afforded the pure maltooligomers which was used for the experiments. By employing maltose and the above single oligomers (glucose units: 3-6) it was investigated whether these substances were substrates of Aspergillus oryzae α-amylase. The time-change of the reducing power values is shown in Fig. 1. (The reducing power is expressed in maltose-equivalents independently of the size of the maltooligomers present in the reaction mixture.)

Table I
Distribution of Maltooligomers at the Hydrolysis of γ-cyclodextrin by Aspergillus oryzae α-amylase at $p_H$ 5.2 and 37°C

| products % / time (min) | glucose | maltose | triose | tetra-ose | penta-ose | hexa-ose | hepta-ose | octa-ose | γ-CD |
|---|---|---|---|---|---|---|---|---|---|
| 20 | 05 | | 26 | 15.7 | 9.1 | 42.9* | 26 | 3.1 | * |
| 30 | 0.58 | | 3.7.7 | 19.9 | 9.8 | 26.9* | 24 | 26 | * |
| 60 | 2.85 | | 7.0 | 19.7 | 4.6 | 2.86 | nd | nd | nd |
| 90 | 3.98 | | 8.25 | 10.3 | 3.1 | nd | nd | nd | nd |

* hexaose and γ-CD together

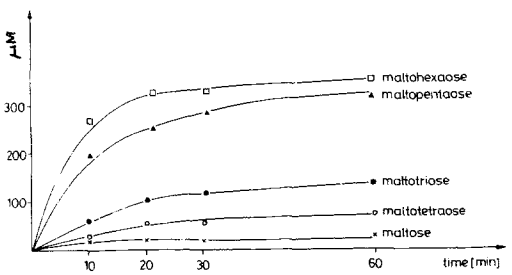



Fig. 1
Reducing power of the reaction products at the hydrolysis of maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose catalyzed by Aspergillus oryzae α-amylase at $p_H$ 5.2 and 37°C. Enzyme concentration $1.62 \times 10^{-6}$ M.

The $V_{max}$ values are summarized in Table II.

Table II
The $V_{max}$ Values at the Hydrolysis of Maltooligomers catalyzed by Aspergillus oryzae α-Amylase

| substrate / $V_{max} \cdot 10^3$ (min$^{-1}$) | 10 min |
|---|---|
| MALTOTRIOSE | 2.79 |
| MALTOTETRAOSE | 1.21 |
| MALTOPENTAOSE | 11.17 |
| MALTOHEXAOSE | 18.15 |

The products formed upon the hydrolysis were examined by means of thin layer chromatography and the results are presented in Table III.

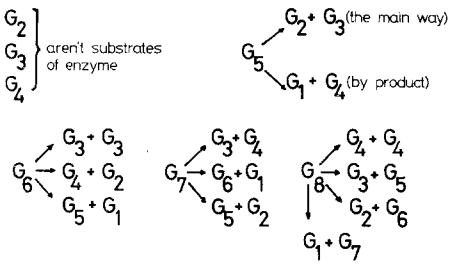
Table III
The Hydrolysis Products of Maltooligomers in the Enzymatic Degradation by Aspergillus Oryzae α-Amylase (The data refer to hydrolysis of 30 minutes.)

| product / substrate | $G_1$ | $G_2$ | $G_3$ | $G_4$ | $G_5$ | $G_6$ |
|---|---|---|---|---|---|---|
| MALTOSE | – | + | – | – | – | – |
| MALTOTRIOSE | * | * | + | – | – | – |
| M.TETRAOSE | * | * | – | + | – | – |
| M.PENTAOSE | + | + | + | * | – | – |
| M.HEXAOSE | + | + | + | * | – | – |

*very little amount

Based on these results the assumed pathways of the degradation are demonstrated by Table IV.

Table IV
Supposed Degradation of Maltooligomers in the Enzymatic Hydrolysis by Aspergillus oryzae α-Amylase




The inhibitory effect of maltose, maltotetraose and maltopentaose was also investigated in the hydrolysis of γ-cyclodextrin with Asperigillus oryzae α-amylase. The results are shown on Figures 2, 3 and 4.

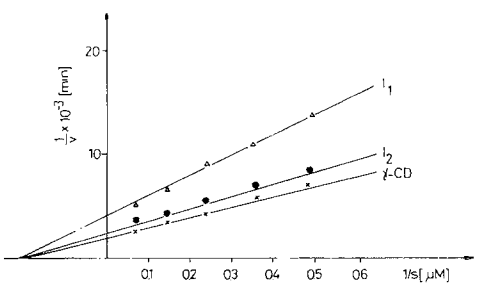



Fig. 2
1/v versus 1/s plots for the hydrolysis of γ-cyclodextrin catalyzed by Asp. oryzae α-amylase in the presence of maltose as an inhibitor. Enzyme concentrations $1.62 \times 10^{-6}$ M. Maltose concentration: $I_1$ 0.47 μmol; $I_2$ 2.34 μmol.

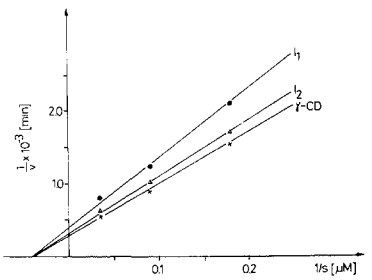



Fig. 3
1/v versus 1/s plots for the hydrolysis of γ-cyclodextrin catalyzed by Asp. oryzae α-amylase in the presence of maltotetraose as an inhibitor. Enzyme concentration $1.62 \times 10^{-6}$ M. Maltotetraose concentration: $I_1$ 0.13 μmol; $I_2$ 0.48 μmol.

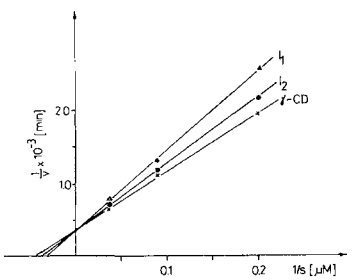



Fig. 4
1/v versus 1/s plots for the hydrolysis of γ-cyclodextrin catalyzed by Asp. oryzae α-amylase in the presence of maltopentaose as an inhibitor. Enzyme concentration $1.62 \times 10^{-6}$ M. Maltopentaose concentration: $I_1$ 0.096 μmol; $I_2$ 0.29 μmol.

According to these data it can be established that maltose, maltotriose and maltotetraose are not good substrates of Aspergillus oryzae α-amylase whereas maltopentaose and maltohexaose act as good substrates of the enzyme, hexaose is the better one.

It is supposed that the enzymatic degradation of maltopentaose proceeds on two ways:

a./ $G_2 + G_3$ (main path)
b./ $G_1 + G_4$ (side reaction)

There are several assumed ways, also of the degradation of maltohexaose:

a./ $G_3 + G_3$
b./ $G_2 + G_4$ (side reaction)
c./ $G_1 + G_5$ (where maltopentaose further decomposes through the paths indicated above).

In the case of maltose and maltotetraose the inhibitory studies established non-competitive inhibition. The

dissociation constant of the enzyme-substrate complex is not affected by the lower-membered maltooligomers, thus the $K_M$ value is constant in a given reaction but $V_{max}$ decreases because of the effect of the inhibitors.

Maltopentaose competitively inhibits the degradation of γ-cyclodextrin with Asp. oryzae α-amylase. Due to its similar structure this oligomer occupies the substrate-binding sites of the enzyme and thus the dissociation constant of the enzyme-substrate complex is changed; the $K_M$ value is increasing and $V_{max}$ remains constant.

During our experiments each maltooligomer including also maltooctaose could be detected and isolated in homogenous form from the hydrolysate of γ-cyclodextrin with Asp. oryzae α-amylase. By freezing the enzymatic reaction at a given time it is possible to obtain the required maltooligomer in an optimum yield and homogenous form suitable for enzyme kinetic examinations. These pure maltooligomers may be utilized also in preparative carbohydrate chemistry as the carbohydrate moieties of artificial antigens or - in suitable derivatized form - for employment as chemical diagnostic agents. Such experiments are now being in progress

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References

1. Green, D.E., Stump, P,K., J. Biol. Chem., (1942) 142, 355
2. Thoma, J.A., Koshland, D.E., J. Am. Chem. Soc., (1960) 82, 3329
3. Numate, C., Thesis of Bachelor's Degree, University of Osaka Prefecture, (1970)
4. French, D., Advances in Carbohydr. Chem., (1957) 12, 189
5. Marshall, J.J., Miwa, T.: Biochem. Biophys. Acta, (1981) 661, 142
6. Abdullah, M., French, D., Robyt, J.F., Arch. Biochem. Biophys., (1966) 114, 595
7. Ben-Gershom, E., Leibowitz, J., Enzymologia, (1958) 20, 133
8. Keay, L., Starch/Stärke, (1970) 22, 153
9. Hanrahan, V.M., Caldwell, M.L., J. Am. Chem. Soc., (1953) 75, 2191
10. Suetsugu, N., Koyama, S., Takeo, K., Kuge, T., J. Biochem., (1974) 76, 57

11. Robyt, J., French, D., Arch. Biochem. Biophys., (1963) 100, 451
12. Robyt, J., French, D., Arch. Biochem. Biophys., (1964) 104, 338
13. Jodál, I., Kandra, L., Harangi, J., Nánási, P., Szejtli, J., Starch/Stärke, (1984) 36, 140
14. Somogyi, M., J. Biol. Chem., (1945) 160, 61
15. Hunt, B.F., Rigby, W., Chem. Ind. (London) (1967) 1868
16. Zemplén, G., Ber. (1926) 59, 1254