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Effects of Native and Permethylated Cyclodextrins on the Catalytic Activity of L-Tryptophan Indole Lyase

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Abstract

The impact of native and permethylated α-, β-, and γ-cyclodextrins on the L-tryptophan indole lyase-catalyzed decomposition of L-tryptophan was investigated by means of spectrophotometric measurements. The inhibitory effects of cyclodextrins on the catalytic activity of the enzyme are shown. The observed inhibition is of mixed type, i.e. competitive and non-competitive. This phenomenon is supposed to be the result of host–guest complex formation involving cyclodextrins and L-tryptophan, and probably between aromatic amino acid residues on the surface of the investigated enzyme. Therefore cyclodextrins were found to have an impact on the maximal velocity and on the Michaelis constant of the described catalysis. The competitive inhibition does not only depend on the stabilities of inclusion complexes, but mainly on their structures.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides with 6 (α-CD), 7 (β-CD) and 8 (γ-CD) D-glucose units, respectively [1]. The physical shape of these molecules resembles that of a torus with a centrally situated cavity. CDs include the hydrophilic surface and the hydrophobic cavity. Owing to these properties, CDs are able to form inclusion complexes with a wide range of compounds [1–3]. The binding forces between the CDs and the guest molecules are not provided by formal chemical bonds [4].

The host–guest complexes of CDs with aromatic compounds including aromatic amino acids are well known [5]. Also, the complexes between native CDs and L-tyrosine, L-phenylalanine, or L-tryptophan have been described in the literature [6–8].

In this paper we present the influence of native cyclodextrins: α-CD, β-CD and γ-CD and also their permethylated derivatives, i.e. per(2,3,6-tri-*O*-methyl)-α-, β-, γ-cyclodextrin (TM-α-CD, TM-β-CD, TM-γ-CD, respectively) on the catalytic activity of L-tryptophan indole lyase (L-Tryptophan indole lyase EC 4.1.99.1, TPase). The present study is a continuation of our previous work focused on the effects of modified cyclodextrins on the catalytic activity of tyrosine phenol lyase [9].

The enzyme tryptophanase, TPase, catalyses the decomposition of L-tryptophan to the corresponding indole, pyruvic acid, and ammonia [10] (Scheme 1). Under certain conditions, there is a possibility of the reverse reaction, leading to formation of L-tryptophan. This enzyme also decomposes L-serine, L-cysteine, and *S*-methyl-L-cysteine, and is often used in the synthesis of L-tryptophan [11]. In the literature, the mechanism of TPase catalysis is well documented [12, 13].

The possibility of forming host–guest complexes between native and modified cyclodextrins and aromatic amino acids encouraged us to investigate the effect of cyclodextrins on the activity of TPase. The influence of selected cyclodextrins on TPase-catalysed reaction was studied based on model decomposition of L-tryptophan. We expected to observe the inhibition of this process due to inclusion complexes formation with amino acid, or conversely, the activation of this process due to formation of inclusion complexes with the product of the enzymatic reaction, i.e. with indole. So, by solving this problem, we could find the proper use of CDs in the enzymatic synthesis of L-tryptophan, but the problem still requires further investigations.

Experimental section

Materials

TM-α-, β-, γ-CD were synthesized using the reported procedures [14], and crude products were purified by means of classic column chromatography on silica gel using the following eluents: chloroform/methanol 10:1

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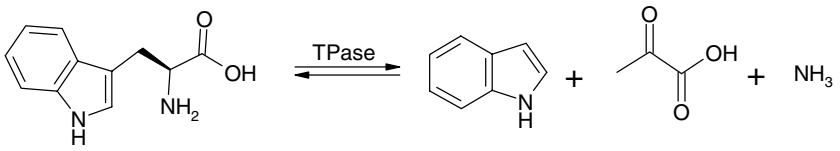


Scheme 1. Decomposition of L-tryptophan catalysed by TPase enzyme.

(v/v) for TM-α-CD and TM-β-CD and chloroform/acetone 2:1 (v/v) for TM-γ-CD.

α-, β-, γ-CD, TPase (L-Tryptophan indole-lyase EC 4.1.99.1) from *Escherichia coli*, L-tryptophan, 5′-pyridoxal phosphate (PLP), dithiothreitol, L-lactic dehydrogenase (LDH EC 1.1.1.27) from *Rabbit muscle*, NADH were purchased from Sigma.

Methods of determination of kinetic data

L-Tryptophan for measurement was preincubated with proper excess of each CD derivative. Incubation was carried out at room temperature overnight. A typical assay contained 0.1 M potassium phosphate buffer, pH 8, 0.2 M dithiothreitol, 50 μM 5′-pyridoxal phosphate, 20.3 U/ml L-lactic dehydrogenase, 0.1 mM NADH, 0.01 U/ml TPase, various amounts of the particular CD and TM-CD (from 1 to 25 mM) and various amounts of L-tryptophan (from 0.4 mM to 10 mM). Six assays of different concentrations of L-tryptophan were applied per one experiment. The decrease of absorbance was measured at a wavelength of 340 nm on a 1202 Shimadzu UV–VIS spectrophotometer during the time of reaction (the kinetics was determined from the change in absorption of NADH at this wavelength). The experiments were performed at room temperature (18 °C). The Lineweaver–Burk plots were used to determine the intercepts (inverse maximal velocity), and slopes (ratio of Michaelis constant per maximal velocity) using the least-squares method. The correction for the decomposition of NADH was added.

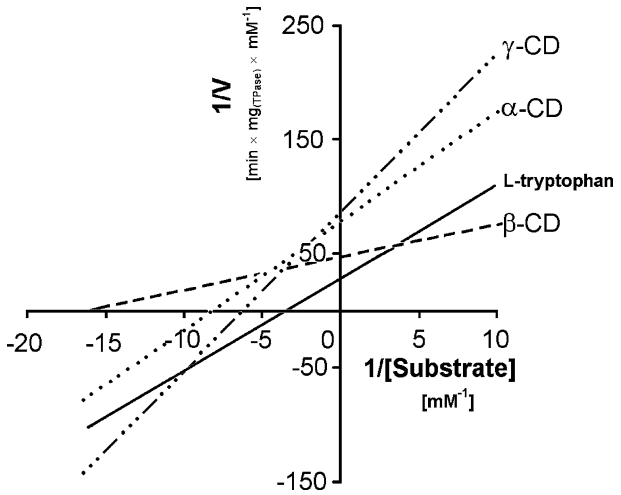


Figure 1. Lineweaver–Burk plot of TPase-catalysed decomposition of L-tryptophan in the presence and absence of particular CD. The obtained plot represents average results for all investigated concentrations of CDs.

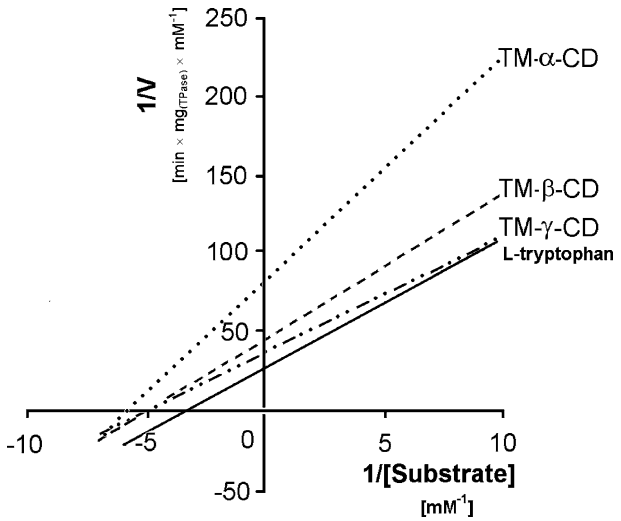


Figure 2. Lineweaver-Burk plot of TPase-catalysed decomposition of L-tryptophan in the presence and absence of particular TM-CD. The obtained plot represents average results for all investigated concentrations of TM-CDs.

Results and discussion

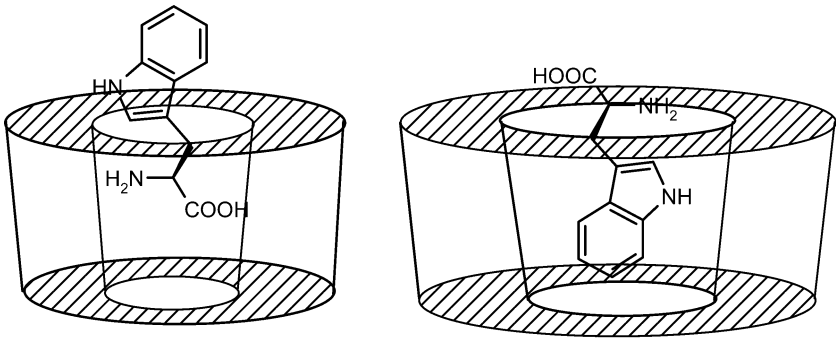
The influence of native and permethylated CDs on TPase-catalysed L-tryptophan decomposition was investigated in the presence and in the absence of these potential inhibitors (CDs) (Figures 1 and 2). In order to prevent CDs binding to the enzyme before the enzymatic reaction, the L-tryptophan was preincubated with appropriate native and permethylated CDs to form inclusion complexes with the substrate. It is obvious that all the investigated CDs could also form inclusion complexes with the product of the described enzymatic reaction, i.e. with indole, but in this case the formation of such complexes was difficult due to the limited time of the reaction.

The stoichiometry of the complex of α-CD with indole is 2:1 [15], but for β-CD is 1:1 [16]. The internal diameter of the cavity in β-CD enables indole to accommodate almost fully, but in the case of α-CD indole only partially enters the cavity. The β-CD-indole complex is also stabilized by hydrogen bond between N-1 hydrogen of indole and either a primary or a secondary hydroxyl group of β-CD [16]. It is difficult to compare the stability of the two above-described host–guest complexes because of their different stoichiometries, but their stability constants for host molecules are as follows: $\log K = 7.8$ (α-CD) [6] and $K = 184$ (β-CD)

[16]. On the other hand, the stoichiometry of the complexes between L-tryptophan and all native CDs is 1:1 and the strongest inclusion complex gives β-CD ($\log K = 3.26$) whereas α- and γ-CD form much weaker complexes ($\log K = 1.79$ and 1.95, respectively) [17]. β-CD has the best internal diameters for the strongest association with L-tryptophan and also with indole. Yang and Bohne [18] established the position of L-tryptophan into the cavity of β-CD in their inclusion complex by a comparison of the size of these molecules. They propose that indole moiety of L-tryptophan is situated fully into cavity of β-CD and aliphatic chain of amino acid with functional groups is outside of host molecule and near of a wider rim of β-CD. Other authors described the α-CD- L-tryptophan complex and claimed that aromatic condensed rings of L-tryptophan accommodate only partially into the cavity of host molecule, yet deeply enough to form hydrogen bonds between ammonium and carboxylate groups of the amino acid and the secondary hydroxyl groups in α-CD. Moreover, the aliphatic chain of L-tryptophan could penetrate the molecular whole of α-CD, though this orientation is approximately 2 kcal/mol less stable than the opposite orientation, i.e. when aliphatic chain is outside the cavity of α-CD [19]. According to above-described properties of complexes between L-tryptophan and α- and β-CD, it is suggested the structures of them, as it is shown in Figure 3.

It is seems that one of the simplest ways of modification of CDs is their permethylation, because alkylation of CDs is an easy one pot reaction. Permethylated CDs have longer cavities with much more increased hydrophobicity of CD edges. In addition to the above-described changes in the properties of permethylated CDs, these derivatives have significantly higher values of solubility in water than their native forms [20–23]. All the described advantages of permethylated CDs encouraged us to apply them in our present study.

The obtained kinetic parameters of catalytic decomposition of L-tryptophan in the presence of all native and permethylated CDs have proved the possibility of formation of inclusion complexes not only with the substrate, but also with TPase (Table 1). We have observed for each of CDs the inhibition of enzymatic decomposition of L-tryptophan. This inhibition is of mixed type, i.e. competitive and non-competitive. The terms of competitive and non-competitive inhibition as well as kinetic equations referring to these processes are explained in Schemes 2 and 3. All the presented



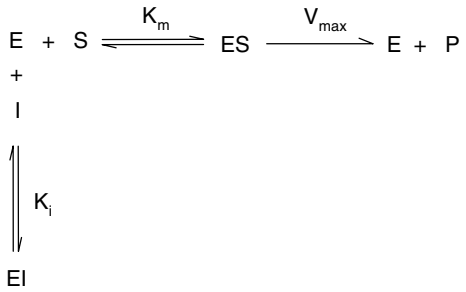
1 **2**

Figure 3. Probable structure of complexes of α-cyclodextrin (**1**), and β-cyclodextrin (**2**) with L-tryptophan.

Table 1. Values of inhibition constants (K_i) for all native CDs and TM-CDs studied in TPase-catalysed reaction as well as Michaelis constants and inverse maximal velocities in the presence (K'_m and $1/V'_{max}$, respectively) and absence (K_m and $1/V_{max}$, respectively) of particular native CDs and TM-CDs

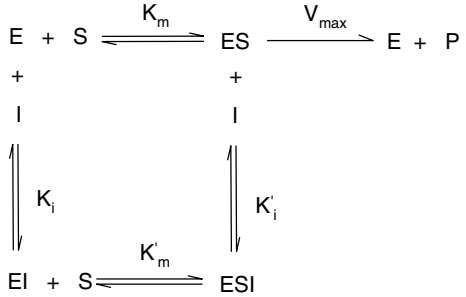
	K'_m [μM]	$1/V'_{max}$ [min × $mg_{(TPase)}$ × mM^{-1}]	K_i^1 [mM]	K_i^2 [mM]
α-CD	817 ± 45	80.0 ± 6.1	9.87 ± 1.27	2.23 ± 0.47
β-CD	1618 ± 242	50.2 ± 3.5	2.47 ± 0.36	8.16 ± 1.31
γ-CD	596 ± 35	90.9 ± 7.2	5.16 ± 0.65	6.22 ± 0.79
TM-α-CD	595 ± 35	84.7 ± 17.7	2.96 ± 0.43	0.50 ± 0.11
TM-β-CD	492 ± 49	47.3 ± 5.2	1.17 ± 0.42	2.25 ± 0.89
TM-γ-CD	506 ± 45	39.8 ± 2.3	1.71 ± 0.37	3.15 ± 0.58
	K_m [μM]	$1/V_{max}$ [min × $mg_{(TPase)}$ × mM^{-1}]		
L-tryptophan	347 ± 27	26.5 ± 3.4	–	–

K_i^1 indicates competitive inhibition; value obtained from the equation: $K_i^1 = [I]/(K'_m/K_m - 1)$; where [I] is inhibitor concentration; K_m is the Michaelis constant; K'_m is the Michaelis constant with presence of inhibitor.
K_i^2 indicates non-competitive inhibition; value obtained from the equation: $K_i^2 = [I]/(V'_{max}/V_{max} - 1)$; where V_{max} is the maximal velocity of the reaction; V'_{max} is the maximal velocity of the reaction with presence of inhibitor.



Scheme 2. Competitive inhibitors (I) usually bind to the active site of free enzyme (E) and in this way they prevent the substrate (S) to bind to the enzyme. It is also another possibility, i.e. some competitive inhibitors can bind to the substrate and therefore they make enzymatic reaction difficult. The K_i is the dissociation constant for the EI complex. The velocity rate of the enzymatic reaction (V') in the presence of the competitive inhibitor depends on the concentration of inhibitor [I] and K_i. Then the Michaelis constant (K'_m) of enzymatic reaction in the presence of competitive inhibitor is derived from the equations [24]: $K_i = \frac{[E]\cdot[I]}{[EI]}$, $K_m = \frac{[E]\cdot[S]}{[ES]}$, $[E]_{0(total)} = [E]_{(free)} + [ES] + [EI]$, $\frac{K_m}{[S]} = \frac{[E]_0}{[ES]} - 1 - \frac{[EI]}{[ES]}$, $\frac{V_{max}}{V} = \frac{[E]_0}{[ES]}$, $\frac{V_{max}}{V} = \frac{K_m}{[S]} + 1 + \frac{K_m\cdot[I]}{K_i\cdot[S]}$ $\frac{V_{max}}{V} = \frac{[S]}{K'_m+[S]}$ $K'_m = K_m(1+\frac{[I]}{K_i})$ where: V_{max} is the maximal velocity of the reaction; K_m is the Michaelis constant with absence of inhibitor.

Lineweaver–Burk plots (Figures 1 and 2) for each cyclodextrin differ from those obtained without their presence. In order to explain this behaviour, we suggest that two kinds of complexes were formed: L-tryptophan–appropriate cyclodextrin and also Tpase–CD complexes. The latter ones could be formed between aromatic amino acids on the surface of TPase.



Scheme 3. Non-competitive inhibitors (I) can bind to the both molecular individualities: enzyme (E) and the enzyme–substrate complex (ES). Such situation is rarely observed for single substrate reactions, but it is common in multiple substrate systems. When the ternary complex ESI is formed, then inhibitor must bind to another site than to the active site. If we assume that the two values of Michaelis constants, K_m and K'_m, are the same, then the maximal velocity (V'_{max}) of enzymatic reaction in the presence of non-competitive inhibitor is derived from the equations [24]: $[EI] \cong [EI] + [ESI]$, $K_i = \frac{([E]_{(free)}+[ES])\cdot[I]}{[EI]}$, $[E]_{0(total)} = [E]_{(free)} + [ES] + [EI]$, $K_i = \frac{([E]_0-[EI])\cdot[I]}{[EI]}$ $\frac{V}{V'} = \frac{[E]_0}{[E]_0-[EI]}$, $[EI] = \frac{[E]_0\cdot[I]}{K_i+[I]}$ $\frac{V}{V'} = \frac{[E]_0}{[E]_0-\frac{[E]_0\cdot[I]}{K_i+[I]}}$ $\frac{V}{V'} = \frac{1}{1-\frac{[I]}{K_i+[I]}}$, $V = \frac{V'_{max}\cdot[S]}{K_m+[S]}$ $V'_{max} = \frac{V_{max}}{1+\frac{[I]}{K_i}}$ where: K_i indicates non-competitive inhibition and is the dissociation constant for the EI complex; V is the reaction rate in the absence of inhibitor; V' is the reaction rate in the presence of non-competitive inhibitor; V'_{max} is the maximal velocity of the reaction with presence of non-competitive inhibitor; V_{max} is the maximal velocity of the reaction with absence of inhibitor; K_m is the Michaelis constant.

The non-competitive inhibition constants for each CD are listed in Table 1 and are in the sequence: β-CD > γ-CD > α-CD for native cyclodextrins and for permethylated derivatives in the sequence: TM-γ-CD > TM-β-CD > TM-α-CD. This suggests that the strength of cyclodextrins binding to the enzyme is in the same order. The plots representing dependence of the inverse maximal velocities in presence of CDs and TM-CDs *versus* their concentrations are shown in Figures 4 and 5.

In this study, 'pseudocompetitive' inhibition seems to be a better name than 'competitive' inhibition because there was competition involved for the substrate between the particular CD and TPase. The values of competitive inhibition constants are listed in Table 1, and are in the following order: α-CD > γ-CD > β-CD and TM-α-CD > TM-γ-CD > TM-β-CD. These values indicate that native cyclodextrins form stronger complexes with L-tryptophan than their permethylated derivatives. The plots representing dependence of the Michaelis constants in presence of CDs and TM-CDs *versus* their concentrations are shown in Figures 6 and 7.

If we compared only the competitive inhibition constants with the stability constants for complexes between L-tryptophan and native CDs (Table 2), this would cause contradictions. The strongest inclusion complex with L-tryptophan forms β-CD, whereas α- and γ-CD form comparable weak complexes. On the other hand, the lowest value of competitive inhibition for native CDs gives unexpectedly β-CD, whereas the highest value gives α-CD. In order to explain this phenomenon, it is worth considering not only the stabilities of these complexes, but also their 3D geometries

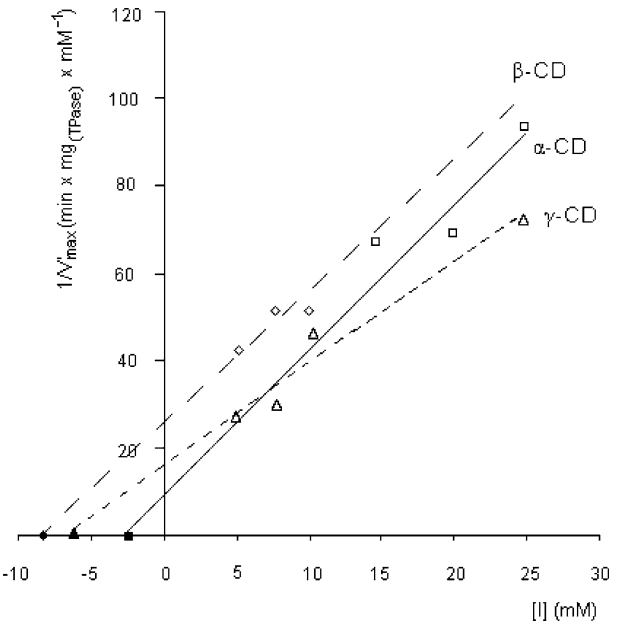


Figure 4. The dependence of the inverse maximal velocities ($1/V'_{max}$) on the concentrations of particular native CD. The experimental points are represented as: □, ◇, △, for α-, β- and γ-CD, respectively. The non-competitive inhibition constants are depicted as the following points: ■, ♦, ▲ for α-, β- and γ-CD, respectively.

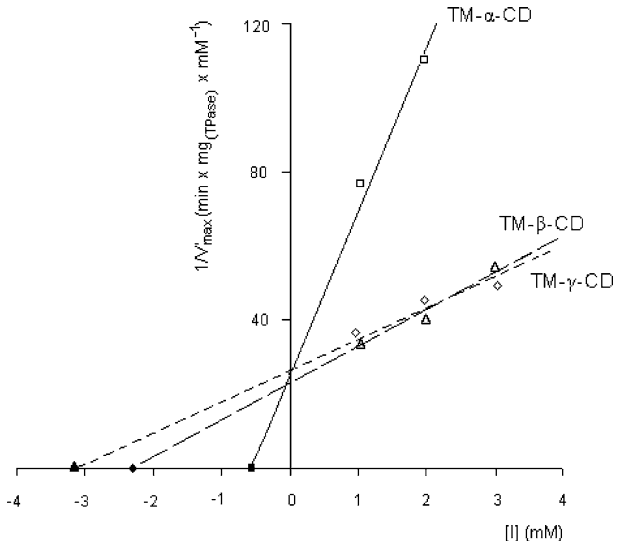


Figure 5. The dependence of the inverse maximal velocities ($1/V'_{max}$) on the concentrations of particular TM-CD. The experimental points are represented as: □, ◇, △, for TM-α-, TM-*β*- and TM-*γ*-CD, respectively. The non-competitive inhibition constants are depicted as the following points: ■, ◆, ▲ for TM-α-, TM-*β*- and TM-*γ*-CD, respectively.

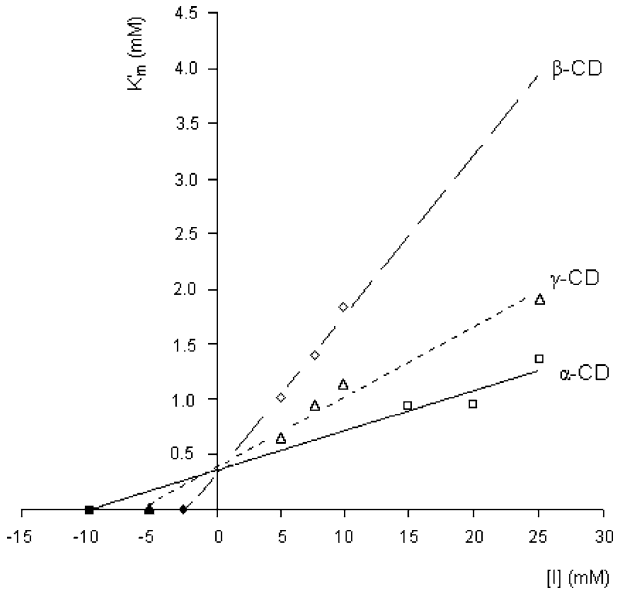


Figure 6. The dependence of the Michaelis constants (K'_m) on the concentrations of particular native CD. The experimental points are represented as: □, ◇, △, for α-, *β*- and *γ*-CD, respectively. The competitive inhibition constants are depicted as the following points: ■, ◆, ▲ for α-, *β*- and *γ*-CD, respectively.

(Figure 3). As it was mentioned above, the aliphatic chain of L-tryptophan could penetrate the molecular whole of α-CD. Such orientation of L-tryptophan in its inclusion complex with α-CD does not enable decomposition of substrate by TPase. Such complex L-tryptophan–α-CD must be unreactive. The high value of competitive inhibition for α-CD confirms the proposed geometry for this complex. Even though the *β*-CD forms much stronger complexes with L-tryptophan, yet in this

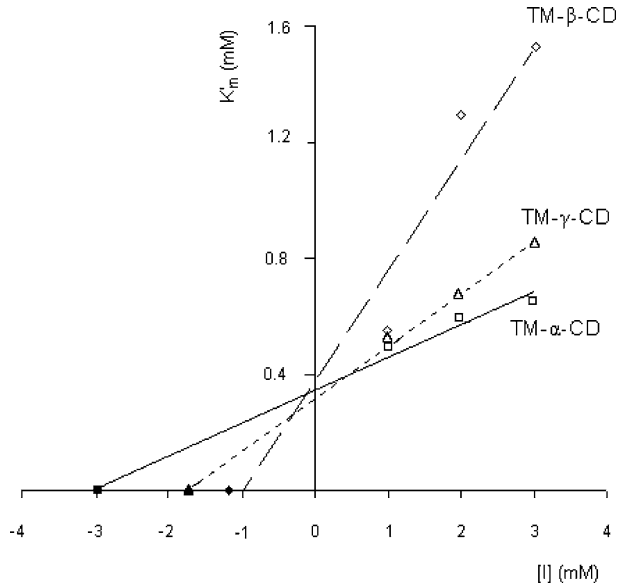


Figure 7. The dependence of the Michaelis constants (K'_m) on the concentrations of particular TM-CD. The experimental points are represented as: □, ◇, △, for TM-α-, TM-*β*- and TM-*γ*-CD, respectively. The competitive inhibition constants are depicted as the following points: ■, ◆, ▲ for TM-α-, TM-*β*- and TM-*γ*-CD, respectively.

Table 2. The comparison of the competitive inhibition constants (K_i^1) with the stability constants (log *K*) for complexes between L-tryptophan and native CDs

	K_i^1 [mM]	log *K*
α-CD	9.87 ± 1.27	1.79[a]
β-CD	2.47 ± 0.36	3.26[a]
γ-CD	5.16 ± 0.65	1.95[a]

[a]Data given from [17].

case the indole group of guest molecule penetrates the 'whole' of host molecule, so the aliphatic chain of L-tryptophan is exposed to the attack of TPase. It indicates that complex L-tryptophan–*β*-CDs are not significantly less reactive than uncomplexed substrate. On the other hand, the internal diameter of *γ*-CD enables the L-tryptophan to accommodate fully in the molecular whole of the host molecule, so the situation for catalytic decomposition by TPase is similar to that of α-CD. Analogous properties for permethylated CDs and native ones are supported by the obtained values for competitive inhibition. The native CDs can better recognise the substrate in Tpase-catalysed decomposition than their permethylated forms.

Conclusions

The presence of a particular native and permethylated cyclodextrin in the course of TPase-catalysed L-tryptophan decomposition has caused the inhibition of mixed type, i.e. competitive and non-competitive. The competitive inhibition depends not only on stability constants of host–guest complexes of L-tryptophan and

CDs, but mainly on their structures. There is the essential condition for TPase-catalysed L-tryptophan decomposition, i.e. the availability of aliphatic chain of L-tryptophan for the enzyme, and it depends on molecular diameters of CDs. α-CD binds L-tryptophan weaker than β-CD, but L-tryptophan penetrates in different ways their cavities, so the competitive inhibition constants are in the direction α-CD > β-CD. The competitive inhibition constants for either α-, β-, γ-CDs or their permethylated forms have been in the same directions and it could be explained by analogical diameters of their molecules.

On the other hand, all investigated CDs caused the non-competitive inhibition in a specific unknown way. It could be explained by forming complexes of CDs with aromatic amino acids on the surface of TPase. Such complexes could cause changes in conformation of enzyme and inhibit enzymatic activity of TPase in this way.

Acknowledgements

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