Effects of water and alcohol on the formation of inclusion complexes of *d*-limonene and cyclodextrins

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Systematic studies have been carried out on the role of water and alcohol in the formation of inclusion complexes between *d*-limonene and α-, β- and γ-cyclodextrin (CD) by a micro-aqueous method. The inclusion complex was barely formed at zero water content for all CDs. Above the specific water content for each CD, formation of the inclusion complex correlated well with an equation which was derived on the autocatalytic assumption for the inclusion phenomenon. The inclusion complex correlated well with an equation which was derived on the autocatalytic assumption for the inclusion phrnomenon. The minimum water content, which was defined as 1% of the maximum concentration of the inclusion complex formed, coincided with the number of water molecules inside the cavity of the CD. In the presence of ethanol, a significant amount of the inclusion complex was formed for β- and γ-CD/limonene systems, particularly at lower moisture content. However, for α-CD the inclusion fraction decreased significantly in the presence of ethanol. This means that ethanol inhibits the formation of the inclusion complex between α-CD and *d*-limonene. For other linear alcohols, the formation of the inclusion complex between *d*-limonene and β-CD increased with decreasing alkyl chain length. This suggests that the more hydrophilic and the smaller (in molecular size) the alcohol is, the more enhanced is the inclusion of *d*-limonene to β-CD.

INTRODUCTION

Cyclodextrins (CDs) are homogeneous cyclic molecules composed of six, seven and eight glucose units called α-, β- and γ-CD, respectively. CDs are host molecules, that can include various kinds of guest molecules into their hydrophobic structural cavities to form inclusion complexes. Since the inclusion complex is useful for stabilization of unstable and/or volatile substances during oxidation or exposure to heat or light, CDs are used to complex many food flavours and fragrances in a powder state. CDs are also useful for improving the solubility of medicines. If powdered CD in a dry state is mixed simply with hydrophobic molecules in the absence of water, no inclusion complexes are formed. This means that the presence of water is essential for the formation of the inclusion complexes between CDs and hydrophobic guest molecules. Recently, we have studied the formation of the inclusion complexes by kneading a slurry mixture of β-CD and *d*-limonene at a low moisture content and found that the formation of the inclusion complex was influenced by the initial moisture content.[1] Kamihara *et al.*[2] have also pointed out that formation of the inclusion complexes between β-CD and aromatic compounds are maximal at a moisture content of 24% under pressurized carbon dioxide. Several intermolecular interactions are responsible for the inclusion of the guest molecules into CDs.[3] However, there has been no systematic research dealing with the effect of moisture content on the amount of inclusion complex formed. From a practical point of view in the pharmaceutical use of CDs, it is important to examine the effect of the presence of other organic solvents such as ethanol in aqueous solutions of CDs.[4]

In this study, we focus our attention on the influence of water and alcohol on the formation of the inclusion complexes at lower water contents. The minimum number of water molecules required for including *d*-limonene into α-, β- and γ-CD was measured by a micro-aqueous method.

MATERIALS AND METHODS

Methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol and *d*-limonene were prepared from *Nacalai tesque*. α-, β- and γ-CD were purchased from Ensuiko Sugar

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Refining Co., and were 99, 99 and 99.5% grade, respectively.

Preparation of the inclusion complex powder

The inclusion complex powders of d-limonene with α-, β- and γ-CD were prepared in the following way. CD powder (0.5 g) was weighed in a 15 × 130 mm glass tube. d-Limonene (2 ml) was added and mixed in a vortex mixer. Alcohol was added at this step, when the effect of alcohol addition was investigated. Then, distilled and deionized water was added dropwise from a pipet or a microsyringe, mixing for 1 min. The inclusion complex was suspended in liquid which was incubated in a water bath at 30°C for 24 h. The supernatant was removed with a Pasteur tube and the inclusion complex slurry dried *in vacuo* at 70°C for 15 h. During vacuum drying, d-limonene adsorbed on to the CDs was evaporated.

Gas chromatographic analysis of d-limonene in the inclusion complex

The content of d-limonene in the inclusion complex powder was measured by gas chromatography. Approximately 100 mg of dried powder was weighed in a capped glass tube, and water (4 ml) and chloroform (1 ml) were added in that order. The solution was heated in a heat block at 80°C for 20 min to extract d-limonene into the chloroform phase. During heating, the tube was periodically mixed in a vortex mixer. The concentration of d-limonene in chloroform was analysed by gas chromatography with a flame ionization detector (GC8A, Shimadzu). The separation was done on a Thermon 1500 column (Shimadzu) at 70°C with nitrogen carrier gas.

Correlation equation for d-limonene content in the inclusion complex powder

The inclusion fraction of d-limonene, which is defined as the molar ratio of d-limonene to CD, was correlated by the following equation over a wide range of water contents.

$$\frac{R}{R_{max}} = \frac{1}{1 + a \cdot \exp(-bw)} \qquad (1)$$

$$a = (R_{max} - R_0)/R_0, \qquad b = k\, R_{max}$$

where w is the water content, k a proportional constant, R_0 the inclusion fraction at $w = 0$, and R_{max} the maximum inclusion fraction. Equation (1) could be derived on the assumption that the inclusion phenomenon was regarded as an autocatalytic reaction with respect to water. Parameter b was used to correlate the experimental data. Equation (1) is similar to the autocatalytic reaction equation. If the minimum

water content w_{min} is defined as the water content at which the inclusion fraction R becomes 1% of the maximum inclusion fraction, w_{min} can be defined as:

$$w_{min} = (1/b)\ln(a/99) \qquad (2)$$

RESULTS AND DISCUSSION

Formation of the inclusion complex at various levels of water content

In Figure 1, the molar ratio of d-limonene to CD (inclusion fraction) is plotted against the molar ratio of water to CD. The formation of inclusion complexes between d-limonene and the CDs was significantly influenced by water content in the liquid mixture. The inclusion complex was barely formed at zero water content for all CDs. Up to a molar water content of about 5, the amount of inclusion complex formed was gradually increased as water content increased. At the specific water content for each CD, however, the inclusion fraction increased rapidly and reached a constant value at high moisture content. It was deduced from the constant inclusion fraction at high moisture content that a 1:1 stoichiometry does not hold between each CD and d-limonene, but it is approximately 2:1 for α-CD, 1:1 for β-CD and 1:2 for γ-CD, respectively. The solid lines in Figure 1 are results calculated by equation (1). Except for the low moisture region, experimental results correlated well with equation (1). This means that the inclusion complex is formed autocatalytically with respect to water. A comparatively large deviation from the theoretical curve in the lower water content region might result from the uncertainty of the R_0 value, since R_0 may influence the parameters significantly.

Minimum water content for the formation of the inclusion complex

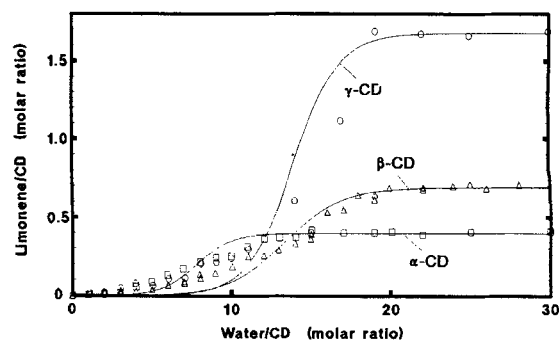The minimum moisture content w_{min} needed for formation of the complex for each CD was calculated

Figure 1 Effect of water content on the formation of inclusion complexes. α-CD ($\square$), β-CD ($\triangle$), γ-CD ($\bigcirc$), calculated results from equation (1) (—).

Table 1 Included water molecules in CD cavities

|	Included water molecules/cavity (molar ratio)	w_{min} by eq. (2) (molar ratio)	Released water molecules/limonene (molar ratio)
α-CD	2	2.72	4
β-CD	7	5.30	7
γ-CD	12	7.35	6

by using equation (2) and the results are listed in the second column of Table 1. The included numbers of water molecules in the first column of Table 1 were determined by crystal analysis.[5] If the inclusion stoichiometries between the CDs and d-limonene are the same as described above (i.e. 2:1, 1:1 and 1:2 for α-CD, β-CD and γ-CD, respectively), the number of water molecules released from the CD cavities by the inclusion of d-limonene can be estimated to be that listed in the third column of Table 1. These values roughly coincide with the minimum number of molecules necessary for the inclusion, w_{min}. If the dried powder is assumed to have no water molecules in the cavities, the water added may be initially included in the CD cavities, and then might be subsequently released because of inclusion of the guest molecule. In such a manner, the inclusion proceeds autocatalytically. According to the inclusion models of Szejtli et al.[6] and Tabushi et al.,[7] the release of the high energy molecules in the CD cavities was postulated for the inclusion. The present results may support experimentally their assumptions.

**Effect of ethanol on the inclusion fraction for each CD**

Extensive studies were made on the effects of the addition of ethanol on the formation of the inclusion complexes between d-limonene and each CD. Figure 2 illustrates the effect of ethanol content on the inclusion fraction for the β-CD/limonene system. In the presence of ethanol, a significant amount of the inclusion complex was formed even when the water content was zero. The most important effect is that in the low moisture region the inclusion fraction increased significantly as the ethanol content increased. However, if the ethanol concentration was higher than 10-fold that of the CD (molar basis), additional enhancement of the inclusion fraction could not be found. The inclusion fraction reached a constant value at the lowest water content as the ethanol content increased. Similar enhancement of the inclusion by the addition of ethanol was found in the γ-CD/limonene/ethanol system, as shown in Figure 3. However, in this case, the rate of increase by the addition of ethanol was less significant than that for the β-CD/limonene system. The solid lines in Figures 2 and 3 are the results
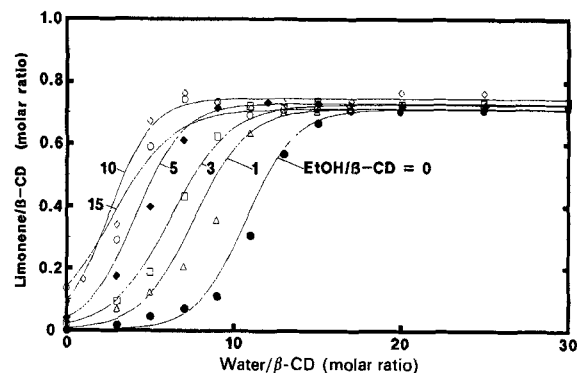
Figure 2 Effect of ethanol on the formation of the inclusion complex between β-CD and d-limonene. Ethanol/β-CD (molar ratio) = 0 (●), 1 (△), 3 (□), 5 (◆), 10 (○), 15 (◯), calculated results from equation (1) (—).
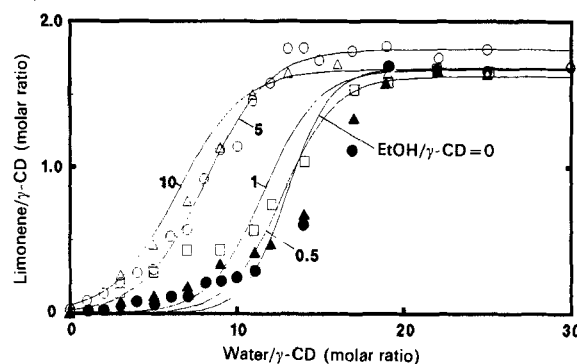
Figure 3 Effect of ethanol on the formation of the inclusion complex between γ-CD and d-limonene. Ethanol/γ-CD (molar ratio) = 0 (●), 0.5 (▲), 1 (□), 5 (○), 10 (△), calculated results from equation (1) (—).
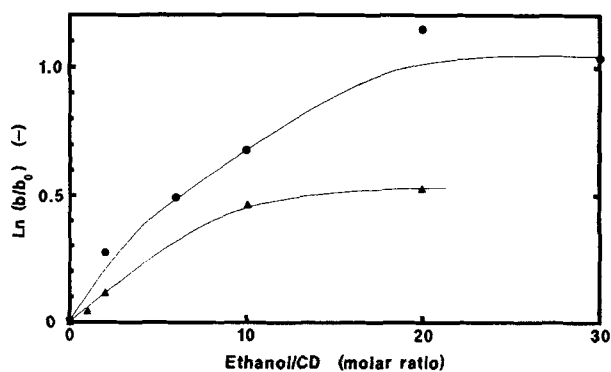
Figure 4 Relationship between parameter b in equation (1) and ethanol content. β-CD (●), γ-CD (▲).

calculated by equation (1). Although the experimental results of the β-CD/limonene system satisfactorily correlate with equation (1), relatively large deviations from the theoretical curves are found in the γ-CD/limonene system because of the scattering of the experimental data. Parameter b was determined so that the best fits were obtained between the calculations and experiments. In Figure 4, parameter b is illustrated

as a function of the molar ratio of ethanol and β-CD. Note that b increases linearly with the amount of ethanol added. However, it is saturated to a constant value at high concentrations of ethanol.

In contrast to the β-CD and γ-CD/limonene/ethanol systems, the results obtained from the α-CD/limonene/ethanol system were quite different, as illustrated in Figure 5. As the molar ratio of ethanol increased, the inclusion fraction decreased significantly, particularly in the high moisture region. It may be considered that ethanol inhibits the formation of the inclusion complex between α-CD and d-limonene. At higher ethanol concentrations, almost all limonene molecules failed to form the inclusion complex with α-CD at water/α-CD > 3. However, at extremely low moisture contents (including zero), formation of the inclusion complex was induced by the addition of ethanol.

Figure 6 shows the content of ethanol in the α- and β-CD/limonene/ethanol complexes with various water contents, together with the inclusion fractions of d-limonene. For the β-CD/limonene/ethanol system, the content of ethanol decreased drastically as the
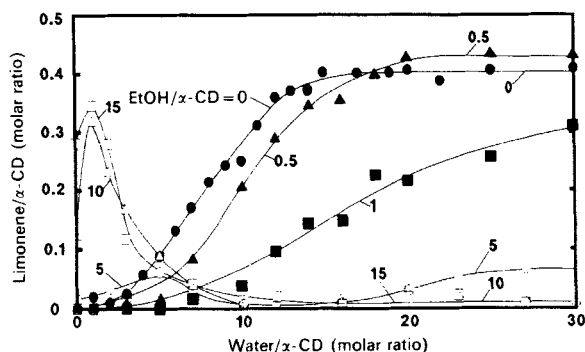

Figure 7 Effect of alcohols on the formation of the inclusion complex between β-CD and d-limonene. Alcohol/β-CD (molar ratio) = 10. Methanol ($\triangle$), ethanol ($\square$), 1-propanol ($\bigcirc$), 1-butanol ($\blacktriangle$), 1-pentanol ($\blacksquare$), no alcohol ($\bullet$).

d-limonene complex increased. This means that ethanol may play a role as a space regulator, as pointed out by Zung *et al.*[8] for the γ-CD/pyrene/ethanol system. On the other hand, the content of ethanol in the α-CD/limonene/ethanol complex remained high even at higher water contents. It can be speculated that d-limonene may be inhibited from forming the complex by ethanol because of the larger molecular size of d-limonene.

Effect of addition of linear alcohols on the inclusion complex

Several linear alcohols were used to examine their role in the formation of the inclusion complex between β-CD and d-limonene. As shown in Figure 7, the inclusion fraction of d-limonene is larger for every alcohol than it is without any alcohols, particularly at low water contents. The inclusion fraction of d-limonene increased with decreasing alkyl chain length. This suggests that the more hydrophilic and the smaller the alcohol is, the more the inclusion of d-limonene into β-CD is enhanced. The solid lines in Figure 7 are calculated results from equation (1), with b being the fitting parameter. The experimental results for the inclusion fraction correlated well with those from equation (1).

Figure 5 Effect of ethanol on the formation of the inclusion complex between α-CD and d-limonene. Ethanol/α-CD (molar ratio) = 0 ($\bullet$), 0.5 ($\blacktriangle$), 1 ($\blacksquare$), 5 ($\bigcirc$), 10 ($\triangle$), 15 ($\square$).

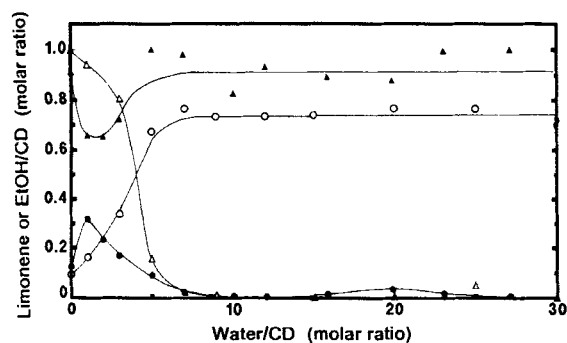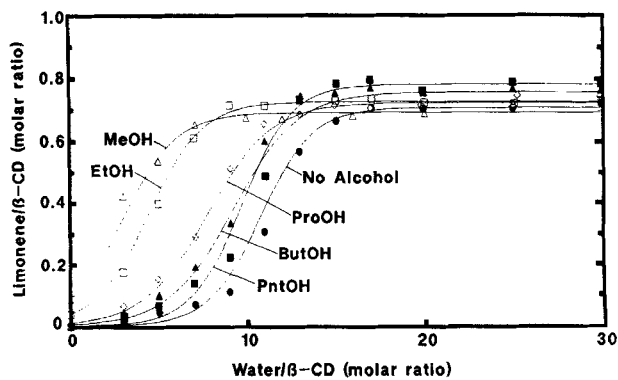**Figure 6** Competitive inclusion between d-limonene and ethanol in α-CD and β-CD. Ethanol/CD (molar ratio) = 10. d-Limonene in α-CD complex ($\bullet$), ethanol in α-CD complex ($\blacktriangle$), d-limonene in β-CD complex ($\bigcirc$), ethanol in β-CD complex ($\triangle$).

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