Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 6, pp. 433-437, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)E0028-Z

Effect of Cyclodextrin on Improvement of Enantioselectivity in the Reduction of Ketopantolactone with Baker's Yeast[1]

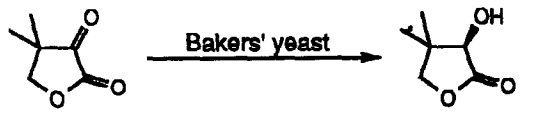
Kaoru Nakamura, Shin-ichi Kondo and Atsuyoshi Ohno*
Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

Abstract—Addition of β-cyclodextrin improves enantioselectivity dramatically in the reduction of ketopantolactone mediated by baker's yeast. It has been found that the selectivity increases with the decrease in concentration of ketopantolactone in bulk solvent, and β-cyclodextrin controls its effective concentration. The role of β-cyclodextrin is discussed.

Introduction

In the last decade, microbial reduction represented by baker's yeast has been applied to syntheses of optically active alcohols.[2,3] Since reduction of a carbonyl compound by yeast does not necessarily afford an alcohol with high enantiomeric excess (*e.e.*), several methods have been proposed to improve the selectivity—modification of substrate,[4–8] addition of specific inhibitor,[9–13] addition of inorganic salt[14] and the use of organic solvent.[15–18] In spite of many such proposals for improving *e.e.* as exemplified above, there still remain tremendous numbers of substrates and reductions that do not give satisfactory results. One of these is the reduction of ketopantolactone (KPL) into pantolactone (PL). Optically active PL is a key intermediate for the synthesis of pantotheic acid, coenzyme A and other biologically active compounds. Both chemical[19–21] and biological[22,23] methods for asymmetric reduction of KPL have been reported. However, chemical reductions so far reported are not satisfactory in enantioselectivity, and biological reductions may require a special microbe which is difficult to cultivate in organic chemistry laboratories.

One of the advantages in using baker's yeast is that this microbe is a cheap and commercially available *reagent* just as other organic reagents. As a part of our effort to explore organic reactions with baker's yeast, we performed asymmetric reduction of KPL with this microbe and found that the addition of β-cyclodextrin (β-CD) improves the enantioselectivity dramatically enough to make the present method practical.[24] We performed detailed studies into the role of β-CD for the improvement of enantioselectivity and the result will be discussed in this paper.



Ketopantolactone (*R*)-pantolactone

Scheme I.

Results and Discussion

Effect of substrate concentration

Although baker's yeast affords the product of desired (*R*)-configuration, *e.e.* is only 73 % when the reaction is run at 20 mM concentration of KPL: the result is not much different from that reported by Lanzilotta *et al.*[25] Since all the methods to perturb enzymes in the microbe previously studied in our laboratory[4–18] failed to afford satisfactory results with the present substrate, we decided to study modification of the environment surrounding the microbe. β-CD is known to form an inclusion complex with various organic compounds using its hydrophobic cavity,[26] and it is highly conceivable that KPL is trapped by β-CD to reduce its effective concentration in bulk solvent.

Indeed, as seen in Figure 1, when initial concentration of the substrate is lowered to 3 mM, apparent increase in *e.e.* up to 96 % is observed even in the absence of β-CD. Thus, there is no doubt that lower concentrations of the substrate result in the improvement of enantioselectivity. Increase in concentration of over 20 mM, on the other hand, exerts no appreciable change in *e.e.* Although we thus found that dilute concentrations of the substrate afford a satisfactory result, 5 mM or lower concentrations are unfortunately too low to employ in the reduction for practical use.

When β-CD is added to the reduction system, the decrease in *e.e.* is less at higher concentrations of the substrate than that observed in its absence. One equivalent or more of β-CD keeps the enantioselectivity constant even at such high substrate concentrations as 20 mM or more. It should be noted that the solubility of β-CD is only 1.4 g/100 mL water (the saturated concentration is 12 mM), and most β-CD molecules added precipitate out of the reduction system.

Concentration of the substrate can also be kept low when it is added to the reduction system dropwise in small portions.[27] When the concentration is kept at either 5 or 2

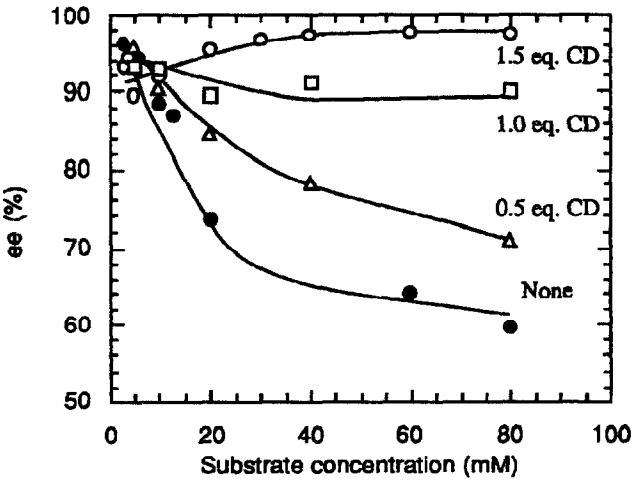


Figure 1. Effect of amount of β-CD on enantioselectivity. Equivalency shown in the figure indicates the β-CD/substrate molar ratio.

mM throughout the reduction by dropwise addition method, the (*R*)-PL is obtained in 91 or 97 % *e.e.*, respectively. Although this method is also effective for obtaining (*R*)-PL in high enantiomeric purity, the reduction requires a tremendously long period and again is unpractical.

Another candidate to control effective concentration of the substrate is the use of an organic solvent.[15–18] Since most organic substrates are hydrophobic, partition between aqueous and organic phases will be much favored by the organic solvent keeping its concentration in the aqueous phase very low. This idea, however, cannot be applied to KPL, because this substrate is highly soluble in water and the partition coefficient, $[KPL]_{benzene}/[KPL]_{water}$, has been measured to be 0.085 ± 0.003 at 30 °C. Thus, KPL prefers the aqueous phase to the organic phase, and when the reduction is run in benzene containing a small amount of water, concentration of the substrate in the vicinity of the microbe in the aqueous phase becomes rather higher than the stoichiometric concentration, giving the worse result of 55 % *e.e.*, compared to 73 % *e.e.* from the reduction in water. The use of ethyl acetate, which dissolves KPL more than water does, in place of benzene improves the result affording (*R*)-PL in 91 % *e.e.* However, again unfortunately, this procedure is associated with another disadvantage: baker's yeast suffers from cell lysis in the presence of ethyl acetate and chemical yield of the product does not exceed about 20 %.

It is thus concluded that the addition of β-CD to the reduction system in water appears to be the best at controlling effective concentration of KPL.

The advantage of low substrate concentration for resulting in satisfactory enantioselectivity of the reduction is made understandable as follows. Since a microbe is a bag of enzymes, there are several reductases in baker's yeast which may produce PL. Some of them will afford (*R*)-PL (*R*-enzymes) while the others afford (*S*)-PL (*S*-enzymes). These enzymes are characterized by their own Michaelis constant, K_m, as one of the kinetic parameters. Since the net result of stereochemistry from the present reduction with baker's yeast has *R*-predominancy, there is no doubt that total activity of the *R*-enzyme(s) overwhelms that of the *S*-enzyme(s) due either to larger quantity or to larger activity or to both. If K_m(s) for the *R*-enzyme(s) is smaller than that(those) of the *S*-enzyme(s), the effective activity of the *R*-enzyme(s) is greater at low concentrations of the substrate, while the activity of the *S*-enzyme(s) is unchanged; thus the predominance of (*R*)-PL is emphasized in the reduction with low concentrations of the substrate.

Indeed, King *et al.* isolated two enzymes from baker's yeast that catalyze the reduction of KPL.[28–31] K_m values for these two enzymes are 14 and 31 μM, respectively, and both afford the (*R*)-PL. No *S*-enzyme(s) has been isolated. We suspect that any *S*-enzyme(s) might have been overlooked by them because of their low activities, or large K_ms. KPL-reductases have also been isolated from other microbes.[32–35] We have isolated seven α-keto ester reductases from baker's yeast.[36] Some of them afford (*R*)-hydroxy esters and others afford (*S*)-hydroxy esters.

The use of cyclodextrin

It is well-known that cyclodextrin includes various organic compounds in its cavity,[26] and so it is suspected that cyclodextrins may be employed as a reservoir of the substrate to keep its concentration in the bulk solvent low throughout the reduction. At the same time, cyclodextrin should permit the survival of baker's yeast in contrast to ethyl acetate (*vide supra*). Thus, the effect of three cyclodextrins (α-, β-, and γ-CDs) on the enantioselectivity was tested, but practically no appreciable effect was recognized with α- and γ-CDs. Inspection with CPK-models reveal that β-CD exerts the best-fit to KPL. Effect of the amount of β-CD on the enantioselectivity is illustrated in Figure 1, and it has been confirmed that the addition of 1.5 eq. amounts of β-CD is satisfactory in producing (*R*)-PL with excellent *e.e.*

It should be noted that the β-CD/substrate ratio is effective in keeping the enantioselectivity excellent and the absolute amount of β-CD exerts no role for the improvement of the selectivity despite the fact that most β-CD molecules stay out of the system as heterogeneous precipitates. This is clearly demonstrated in Figure 2, where *e.e.* of the product, (*R*)-PL, is plotted against substrate concentration keeping the amount of β-CD constant. The enantioselectivity decreases in each run with the increase in concentration of the substrate, which confirms that the molar amount of β-CD should exceed that of the substrate.

Although, at first glance, there seems to be no doubt that β-CD includes KPL in its cavity to reduce the substrate in bulk solvent, this idea is not as straightforward: first of all, KPL is, as mentioned above, not as hydrophobic as other regular organic materials and there is no reason for KPL in the aqueous layer to come preferentially into a hydrophobic cavity. Secondly, β-CD which is dissolved in water (saturated concentration: 12 mM) precipitates immediately when KPL is added to the system. Although we tried to measure the association constant between KPL and β-CD,

no efforts (UV and ^{1}H NMR) have succeeded in obtaining the association constant, because the solubility of the inclusion complex is too small to be measured correctly. On the other hand, when modified β-CDs such as 2-hydroxypropyl-β-CD (HP-β-CD) and maltosyl-β-CD (G2-β-CD) are employed as reservoirs, the improvement of enantioselectivity is much less than for unsubstituted β-CD. These substituted β-CDs are soluble in water even when they include KPL. The effect of substituted as well as unsubstituted β-CDs are illustrated in Figure 3.

In order to understand the role of β-CD, the amount of substrate remaining in the solution after a β-CD is added to an aqueous solution of KPL has been monitored and the results are summarized in Table 1. When 1.5 eq. amounts of unsubstituted β-CD are added to the solution, the concentration of KPL in the solution is reduced to 1/100 of its stoichiometric one. On the other hand, water-soluble HP-β-CD exerts no ability to reduce the concentration.

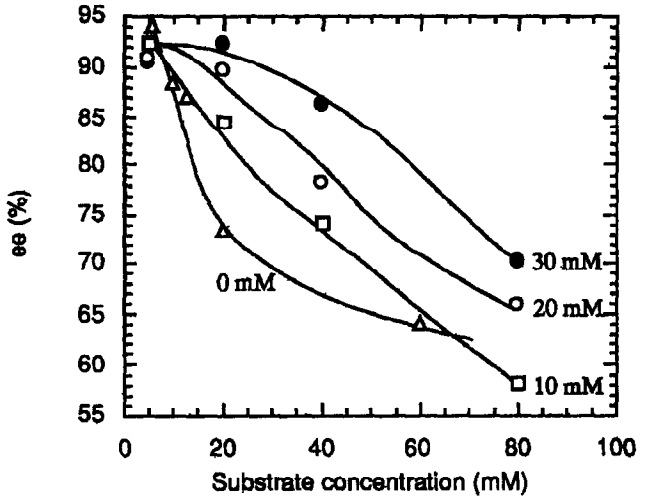


Figure 2. Effect of concentration of β-CD on enantioselectivity. Concentrations shown in the figure represents that of β-CD.

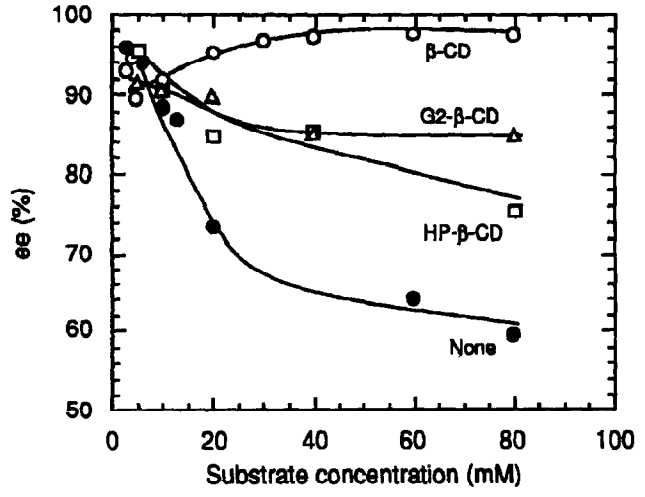


Figure 3. Effect of substituted and unsubstituted β-CDs on enantioselectivity. For abbreviations in the figure, see text.

Table 1. Concentration of Ketopantolactone Remaining in Aqueous Phase in the presence of β-CD

Cyclodextrine[a]	$[KPL]_{initial}$ (mM)	$[KPL]_{aq}$[b] (mM)
β-CD	80	ND[c]
β-CD	20	0.25
β-CD	5	0.66
HP-β-CD	80	58.7
HP-β-CD	20	19.0
HP-β-CD	5	4.92

[a]1.5 eq. amount to KPL.
[b]Errors are less than ± 10%.
[c]Too small to be measured.

The result reveals that the substrate concentration maintained in a solution by free ⇌ inclusion equilibrium is not low enough to afford a satisfactory result when the system is homogeneous. On the other hand, when a part (or, more precisely, most) of the substrate molecules are taken out of the system forming a heterogeneous phase, then another process participates, a solid ⇌ liquid equilibrium, and this heterogeneous equilibrium might be much more effective in reducing the concentration of the substrate in the bulk solvent than the homogeneous free ⇌ inclusion equilibrium. Thus, inclusion as the sole effect is unsatisfactory in explaining such an efficient role of β-CD.

Here, however, the substrate is not necessarily included exactly in the cavity of β-CD. If it is co-precipitated with β-CD, the purpose of keeping the substrate out of the reduction system is efficiently achieved. We claim, therefore, that β-CD acts as a reservoir of KPL when it can stock the substrate outside the reduction system and supplies the substrate on request to the system so that the concentration of substrate in the bulk solvent is effectively low throughout the reduction, whatever their interaction is.

Experimental Section

Instruments

^{1}H NMR spectra were recorded on a Varian VXR-200 spectrometer in $CDCl_3$ with $CHCl_3$ as an internal standard. Gas-liquid chromatograms were recorded on a Shimadzu GC-14A gas–liquid chromatograph.

Materials

Dry baker's yeast was purchased from Oriental Yeast Co., Japan. Ketopantolactone (KPL) was purchased from Aldrich Chemical Co., Inc. Authentic racemic and (*R*)-PL were obtained from Nacalai Tesque Co. Ltd. α- and β-Cyclodextrins were supplied by Hexa Co. Ltd. γ-Cyclodextrin was purchased from Nacalai Tesque Co. Ltd 2-Hydroxypropyl-β-cyclodextrin and maltosyl-β-cyclodextrin were presented kindly by Nihon Shokuhin

Kako Co. Ltd and Ensuiko Sugar Refining Co. Ltd, respectively.

General procedure for reduction of KPL

Baker's yeast (3.0 g) was added to a 15 mL aqueous solution of KPL (38.4 mg, 20 mM). The mixture was shaken at a rate of 130 times/min for two days at 30 °C. After the reaction, acetone (15 mL) was added to the mixture and precipitates were filtered off. The solvent was evaporated from the filtrate under reduced pressure. Residual mixture was extracted with chloroform (30 mL x 2) and combined organic phase was washed with brine. The organic phase was dried over anhydrous magnesium sulfate and filtered. The solvent was evaporated under reduced pressure and residual oil was purified by silica gel column chromatography (eluent, ethyl acetate : hexane = 1 : 2) giving pantolactone (16.0 mg, 41 % yield).

General procedure for reduction of KPL with cyclodextrin

β-Cyclodextrin (510 mg, 0.45 mmol) was added to a 15 mL aqueous solution of KPL (38.4 mg, 0.3 mmol, 20 mM). After the mixture was agitated vigorously, baker's yeast (3.0 g) was added to the mixture. After the procedures described above, PL was obtained in 39 % yield. Reductions with other cyclodextrins were run similarly.

Reduction of KPL in ethyl acetate/water biphasic system

To a suspension of baker's yeast (3.0 g) in water (15 mL), KPL (51.3 mg, 0.4 mmol) in ethyl acetate (15 mL) was added slowly. The heterogeneous mixture was stirred slowly at 30 °C. The extent and enantioselectivity of the reaction were monitored by GLC analysis (PEG 20 M, 25 m x 0.25 mm ID, 160 °C). After 27 h, the reaction proceeded to 21 % compression with *e.e.* of the product of 91 %.

Determination of enantiomeric excess of PL

Enantiomeric excess in the produced PL was determined by gas–liquid chromatography analysis with Chirasil-L-Val (25 m x 0.25 mm ID) capillary column gas–liquid chromatography at 100 °C. Retention times of *S* and *R* enantiomers were 5.9 and 6.6 min, respectively.

Determination of the concentration of KPL in aqueous phase

To each 80, 20 and 5 mM aqueous solution of KPL (12.8 mg), β-cyclodextrin (170.3 mg, 1.5 eq.) was added. After vigorous agitation, the mixture was shaken continuously for 1 h at 30 °C. To 500 μL of an ethyl acetate solution of hexadecane (5.21 mM), 300 μL of each aliquot was added and total mixture was shaken vigorously to extract free KPL into the organic phase. The organic phase was analyzed by gas–liquid chromatography (PEG 20 M, 25 m x 0.25 mm ID, 120 °C) and concentration of KPL was determined by use of hexadecane as an internal standard. Experiments with 2-hydroxypropyl-β-cyclodextrin (179 mg, *ca* 1.5 eq.) were run similarly. The results are shown in Table 1.

Acknowledgement

We thank the Ministry of Education, Japan, Science and Culture under the Grant-in-Aid Nos. 04403006 and 04303002 for financial support for a part of this research. We also thank Ensuiko Sugar Refining Co. Ltd and Nihon Shokuhin Kako Co. Ltd for the kind gift of substituted β-CDs. We appreciate Professor H. Tsukube, Okayama University, for his valuable discussion and suggestion.

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36. Purification and characterization of these reductases are now in progress in our laboratory.

(*Received* 25 *October* 1993; *accepted* 21 *January* 1994)