β-cyclodextrin 및 그 유도체의 포접체 형성에 의한 LG 106W의 유용성 및 안정성 개선에 관한 연구

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Improvement in availability and stability of LG 106W by inclusion with β-cyclodextrin and its derivatives

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Abstract

A newly synthesized polyhydroxy aromatic compound(LG 106W) has good skin lightening effect. Inclusion complexation of LG 106W with β-cyclodextrin and its hydroxypropyl and dimethyl derivatives was studied by the solubility method, scanning electron microscopy and differential thermal analysis. A relationship between host and guest was clearly reflected in the magnitude of the stability constant(DM-β > HP-β > β-cyclodextrin). Formulation problems, which resulted from its very low solubility in aqueous solution, were resolved by the inclusion formation. LG 106W from inclusions is much more water-soluble than pure one. The improvement of pH and temperature stability correlated with the increased solubility was also observed. Inclusion complex of LG 106W had similar activity to pure LG 106W on the inhibition of melanin synthesis in B-16 melanoma cell but showed lower irritation on cultured cell test in vitro. According to the results, cyclodextrins might be one of the reliable candidates for improving the availability of LG 106W.

Introduction

Cyclodextrins(CyDs) are water-soluble, cyclic oligosaccharides consisted of six, seven or eight D(+)glucopyranose units linked by α-1, 4 glycosidic bonds and they are known as α-, β-, and γ-cyclodextrin respectively. Cyclodextrins were first isolated in 1891 as degradation products of starch by the action of cyclodextrin glycosyl transferase.[1] Some of their properties are given in Table 1.

The cyclodextrin molecule is torus shaped with the primary hydroxyl(C6 hydroxyl) at the narrow edge of the torus and the secondary hydroxyls(C2 and C3 hydroxyls) positioned at the wider edge of the torus(Figure 1).[2] The interior cavity of this molecule is hydrophobic while the outer surface of the molecule is hydrophilic. Owing to that, cyclodextrins(hosts) are able

Table 1. Some properties of cyclodextrins.

CyD	Number of glucose units	Molecular weight	Cavity[a] Diameter(Å)	Approximate cavity volume(Å^3)	Solubility[b] (g/100ml)
α-CyD	6	973	5	174	14.5
β-CyD	7	1135	6	262	1.85
γ-CyD	8	1297	8	427	23.2

[a] Estimated by the Corey-Pauling-Koltun model.

[b] In water at room temperature.

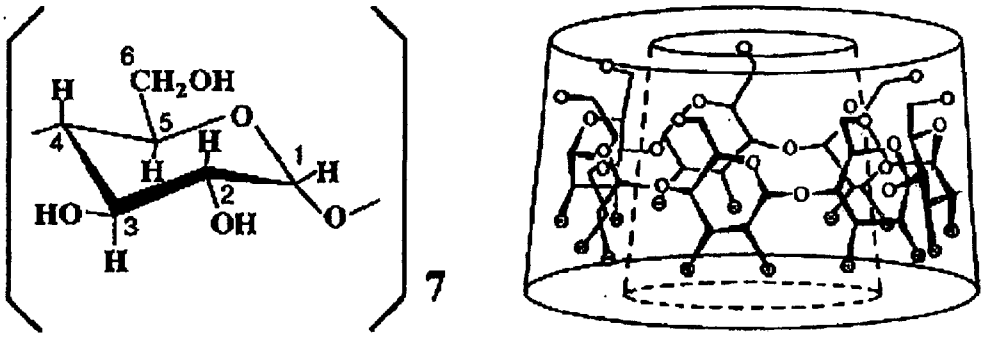

Figure 1. Structure of β-cyclodextrin.

to form inclusion compounds in solution as well as crystalline clathrates with a large variety of molecules(guests).[3, 4] The interaction between the host cavity and the guest molecule causes a structural perturbation of the guest and consequently a modification of its physical, chemical and biological properties. Because of their different internal cavity diameters, each cyclodextrin shows a different degree of molecular encapsulation with different-sized guest molecules. In the cosmetic and pharmaceutical fields, cyclodextrins have been recognized as potent candidates to overcome the undesirable properties of guest molecules through the formation of inclusion complexes.[5, 6]

In the previous paper, we disclosed a newly synthesized polyhydroxy aromatic compound (LG 106W, Figure 2) synthesized through the examining of the structure-activity relationship of the natural polyhydroxy aromatic compounds. Skin lightening effects of this compound were also mentioned. This new compound showed strong inhibitory activity against mushroom tyrosinase and good lightening effects due to the inhibition of melanogenesis.[7] But this lightening agent exhibited very low solubility in water and low availability which, added to its stability, made its formulation very problematic in skin care products.

This study was intended to evaluate the role of cyclodextrin and its hydroxypropyl and dimethyl derivatives towards improving the physicochemical characteristics and availability of LG 106W having good skin lightening effect.

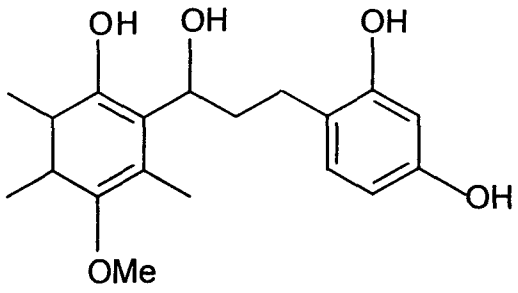

Figure 2. Chemical Structure of LG 106W.

Materials and Methods

Materials

LG 106W(Figure 2) was synthesized by LG Chem. Biotech Research Institute at a purity of

at least 99%, and used without any further purification. Its molecular weight is 332.39 and melting point is 192℃.

The β-cyclodextrin and its hydroxypropyl and dimethyl derivatives were obtained from Wacker-Chemie GmbH(Burghausen, Germany) and employed without any preliminary tests. All other chemicals were of reagent grade.

Solubility studies

Solubility measurements were carried out according to the Higuchi[8] and Lach(1954). Excess amounts of guest compound were introduced into 1.5ml polypropylene microcentrifuge tubes containing various concentrations of cyclodextrins in 0.1M phosphate buffer solution and firmly sealed with parafilm(American Can Co., Greenwich, CT). The tubes were shaken at room temperature for 72 hours. After equilibrium was attained, they were centrifuged at 3,000rpm for 5min(TOMY Micro Centrifuge, MRX-150, Japan). Non-wetted floating particles and non-solubilized guest molecules were eliminated by filtration using a membrane filter(0.2um, MFS-3, Micro Filtration Systems, CA) pre-saturated with the guest compound(to avoid guest adsorption on the filter membrane). A portion of the filtrate was diluted with 50 %, v/v MeOH-water and the solubility were determined by HPLC. The experiments were carried out in triplicate.

The HPLC consisted of solvent delivery pump(Waters 626 pump, Waters Co., MA, USA), C18 column(Hewlett Packard BDS), UV detector(Waters 486 Detector) and data processing system(Millennium, Waters). The mobile phase was 58% MeOH in 0.05% phosphoric acid and the flow rate was 1.0ml/min. Wavelength of 283nm was selected and the temperature of the column was kept at 30℃. The retention time was 3.19min for LG 106W. The correction of concentration against each sample point was undertaken.

An apparent stability constant, Kc, was calculated from the initial straight line portion of phase solubility diagrams according to the following equation[9](Higuchi and Connors,1965). Solubility in some kinds of Polyols was determined in the same way.

$$Kc = \frac{\text{slope}}{\text{intercept} \cdot (1\text{-slope})}$$

Preparation of the inclusion complexes

For the complex preparation, two kinds of methods were available which could be used depending on the physical properties of the guest and cyclodextrins.

In the case of preparation of solid complexes, an aqueous solution of β-cyclodextrin is prepared at room temperature. An equimolar amount of the guest compound is added and the mixture is stirred at ambient temperature for 24 hours. β-cyclodextrin complex formation is indicated by the precipitation of a white solid precipitate. The solid complex is isolated by filtration, washed with distilled water and dried under vacuum.

To prepare the complexes with cyclodextrin derivatives, an excess amount of the guest compound is added to aqueous solution of the cyclodextrin derivatives and the mixture is stirred at ambient temperature for 24 hours. After the excess of guest compound has been removed by filtration, the solid complex is obtained by spray drying the filtrate.

Determination of the physicochemical properties of the complex

The microscopic aspect of the raw materials was compared with that of the products obtained by precipitation and simple physical mixture, by examination under the scanning electron microscope.

The reality of the inclusion was confirmed by subjecting the raw materials, the simple physical mixture and the precipitated product to differential scanning calorimetry. The DSC analysis was performed using a Setaram TG-DSC instrument(Caluire, France). The samples were analyzed in an open capsule to allow the evaporation of water lost by cyclodextrin. Determinations were carried out on 10mg of LG 106W, or on the corresponding quantities. The different samples were heated from 25 to 250℃ at a rate of 10℃/min.

Stability studies

To measure the temperature stability, 40% 1,3-BG solution was prepared and divided into 2 parts. 0.1% LG 106W in free state or complex form was added to the each solution. The solutions were moved to 5.0ml teflon FEP centrifuge tubes(Nalgene, Nalge Company, NY). The tubes were sealed with parafilm and kept at 25, 40, 50℃ in temperature controlling chamber. Periodically, 0.1ml aliquots of those were pipetted out and then diluted with 50%,v/v MeOH. The solubility changes were determined by HPLC.

In the case of pH-stability study, a universal buffer solution was prepared and the experiment was done like the same way of temperature stability test.

Inhibition of melanin synthesis in B-16 melanoma cell

B16 mouse melanoma cells were cultured in DMEM supplemented with 10% fetal calf serum in humidified incubator at 37℃ under 5% CO_2. Cells were seeded into 60 π petri dish at a density of 5×10^5 cells per dish. After cells were attached, medium was replaced with fresh medium containing various concentrations of LG 106W and its cyclodextrin complexes. Then cells were cultured for 2 days and the medium was replaced with fresh medium, further incubated for a day. Then cells were harvested with cell scrapper. The inhibition of melanin synthesis was measured by expert's sight.

Cytotoxicity test

In order to screen the potential toxicity of raw materials, MTT-reduction test was carried out. First, V79-4cells(Chinese Hamster, CCL of lung tissue fibroblast) were cultured and then the serial dilution of the test materials were applicated to the cultured cell for 24 hours. For viability check, MTT-test was performed according to the method previously described. MTT-formazan extracts were measured by reading the absorbance at 570nm.

Results and Discussion

Physicochemical analysis of inclusions

Analysis by the scanning electron microscope(Figure 3) reveal the crystallization of β-cyclodextrin in polyhedral form with relatively large dimensions, whereas LG 106W is substantially in needle shape. The inclusion complex obtained by precipitation occurs in the form of large hexagonal alone or crossed each other and it has relatively smooth surface. This observation, while revealing a clear difference between the precipitated complex crystal and the physical mixture, is inadequate to conclude a genuine inclusion, but helps to assess the existence of an inclusion in the preparations obtained.

Differential thermal analysis was much more informative. β-cyclodextrin exhibits a very broad thermal rise around 67±5℃, corresponding to a release of water. Its degradation,

which takes place at 300℃(caramelization, data is not shown) does not appear on the recording. LG 106W exhibits a thin fusion peak at 192℃. The physical mixture produces a recording more or less the superposition of cyclodextrin and LG 106W. On the other hand, for the inclusion complex, the recording is rather different and is distingushed by the disappearance of the rise due to the loss of water from the cyclodextrin, the disappearance of the LG 106W fusion peak, and the appearance of a new characteristic peak at 202℃. Thus, the temperature shift(about 10℃) between the both proves that some interaction exists between LG 106W and β-cyclodextrin, corresponding to an inclusion formation with weak physical bonds. The existence of this new compound is also confirmed by the disappearance of the water loss peak of cyclodextrin.

Solubility studies

Figure 5 shows the phase solubility diagrams obtained for LG 106W with 3 cyclodextrins, where the difference in solubility curves was clearly noted. The solubilities of LG 106W in 0.1M phosphate buffer solution are significantly increased by inclusion in β-cyclodextrin and its derivatives. Unfortunately, the extent of the concentration range of cyclodextrins done in this experiment was not enough to precisely calculate the stability constant, but based on the solubility diagrams, the following results were attained. In the case of β-cyclodextrin and HP-β-cyclodextrin, they increased linearly as a function of concentrations and the solubility curves can be generally classified as type A_L. On the other hand, the solubility of LG106W with DM-β-cyclodextrin showed a little bit different diagram, where the solubility curve can be classified as A_L coexisted with A_P. The ascending curvature of DM-β-cyclodextrin in the figure was quantitatively analyzed according to the optimization technique previously published in other paper[10] to obtain the stability constant of high-order complexes. The apparent stability constants, Kc, were calculated from the initial straight line portion of the solubility diagrams. The result of solubility study is listed in Table 2.

It is important to determine the Kc value with accuracy, because this parameter is a useful index to estimate the changes in physicochemical properties of the guest molecule in the inclusion. In the result, Kc values increased in the order of DM-β > HP-β > β-cyclodextrin and they generally formed 1:1 complex.

Table 2. Stability Constant of LG106W complexes.

CyDs	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
β-CyD	3,897	-
HP-β-CyD	3,964	-
DM-β-CyD	6,186	2.0

Figure 6 shows the solubility of LG 106W with 3 Polyols(1,3-butylene glycol, propylene glycol, glycerin). This experiment was done preliminary to fix the adequate concentration of polyol for dissolving LG 106W to continue the stability study and to compare the solubility enhancement among polyols. Solubility enhancement increased significantly as the concentration of polyols increased and showed in the order of 1,3-BG>PG>>Glycerine. In skin care products, it might be inadequate to increase the solubility of LG 106W with only increasing the concentration of polyols. Because the solubility of it in aqueous phase is very low with adequate range of polyol concentration(0~20%) and specific concentration point can not be easily expected because of non-linear solubility and other unpredictable components in the system.

In this experiment, 40% 1, 3-BG solution was chosen for the stability test.

Stability studies

Figure 7 [A] shows the stability change of LG 106W in free or complex form with the variety of temperature. This result suggested that the inclusion of LG 106W in HP-β-cyclodextrin conferred protection to it but in a small margin.

From the results of pH stability(Figure 7, [B] and [C]), we can assume that the adequate pH of the complex when it is applied to product is 5.6~7.7, and the sensitivity of LG 106W to acid or base is reduced by inclusion in HP-β-cyclodextrin. On the contrary to the above results, depending on the pH, inclusion can render the active ingredients unstable in aqueous solution in some cases. Szejtli[11] noted that the inclusion of vitamin K_3 in β-cyclodextrin(which improved its heat stability in the solid state[12]) had no effect on its light stability in neutral or slightly acidic solution.

Stability improvement has three essential objectives : heat stability, resistance to oxidation

and resistance to hydrolysis(stability in aqueous solution). Skin care cosmetics in markets almost contain water as a main component, so it is important to check the stability of active compound in aqueous solution and to search tools for improving the stability. In this view point, cyclodextrins might be one of the reliable candidates to improve the stability of LG 106W or any other similar compounds in the product during the shelf life.

For the investigation of the effect of LG 106W and its HP-β-cyclodextrin complexes on B-16 melanoma cells, we fixed the concentration of the active material and only changed the ratio of HP-β-cyclodextrin(5% w/w, 1:1, 1:2 molar ratio). When cultured with 0.02mg/ml and 0.05mg/ml of LG 106W, there was linear decrease in melanin contents of the cells. More than 0.05mg/ml concentration of LG 106W alone, it was difficult to keep the experiment on because of low solubility and cell death. But in the HP-β-cyclodextrin complexes, it was easy to perform the test due to the increase of solubility and we were able to find that the complexes could lower the toxicity on that cell as well.

Table 3. Effect of LG 106W and its HP-β-CyD complexes on B-16 mouse melanoma cells.

LG106W Conc.(mg/ml)	LG106W alone	Complex (5%, w/w)	Complex (1:1)	Complex (1:2)
0.02	+	+	+	+
0.05	++, 20%*	++(+), 40%*	++(+), 40%*	++(+), 40%*
0.10	-	++(+), 20%*	++(+), 20%*	++(+), 20%*

* Cell viability

Considering on the above result, in vitro toxicological test was accomplished to study more about the cell viability. Figure 8 shows the result of that. In this figure, compared to the result of LG 106W alone, the cytotoxicity of LG 106W can be attenuated in the range of 0.0047~0.012% by complex formation regardless of the HP-β-cyclodextrin concentration and it might be one of the good candidates for improving skin safety.

Various investigations have revealed a decrease in the irritant effect of included substances. This means that, by dissimulating part of the molecule inside the cavity of a cyclodextrin, the harmful effects are diminished. For example, Takeda Chemical Industries reported[13] the

harmful effects of the stimulation of the skin by 2-n-octyl-4-octyl-isothizolein-3-one are vastly decreased if the substance is in the form of an inclusion. The irritant effect of perfume can be diminished in the same way.[14, 15, 16]

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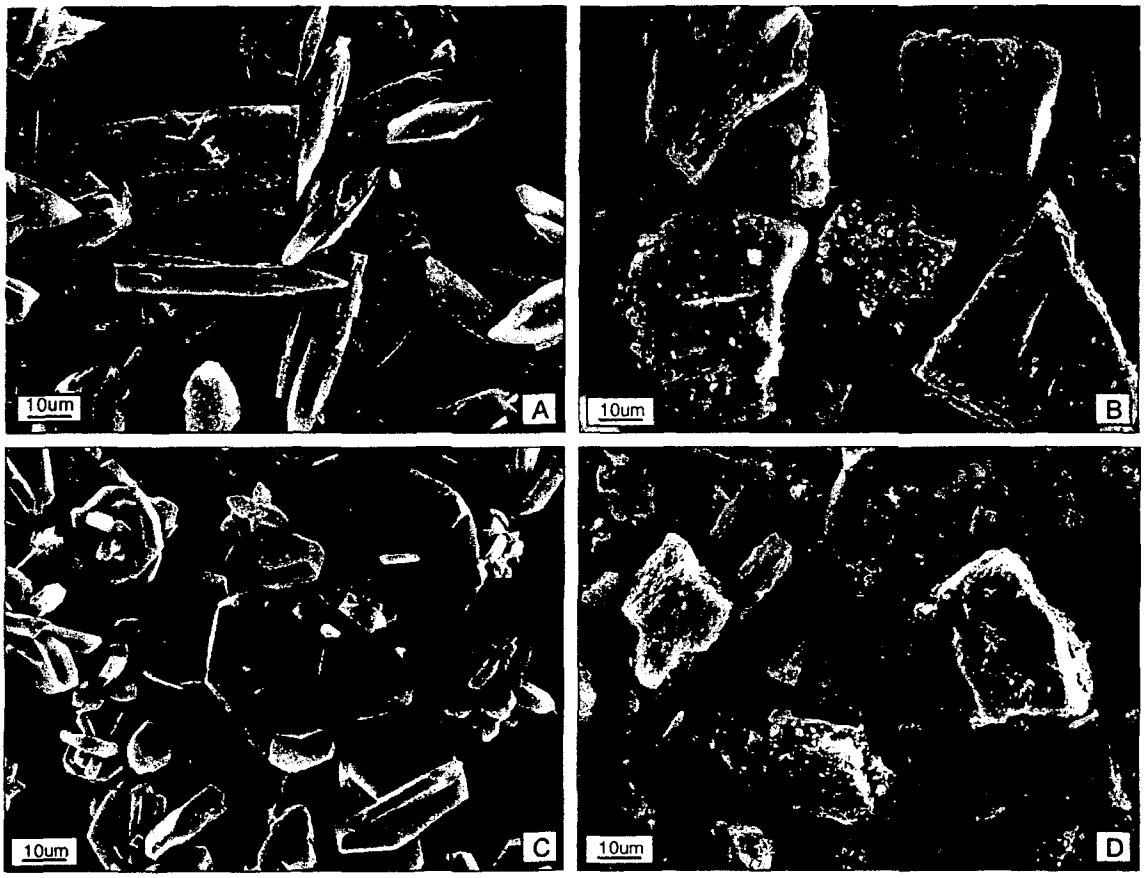

Figure 3. SEM photograph of the surface condition of the particles
(A: LG 106W, B: β-CyD, C: LG106W/β-CyD precipitate, D: Physical mixture).

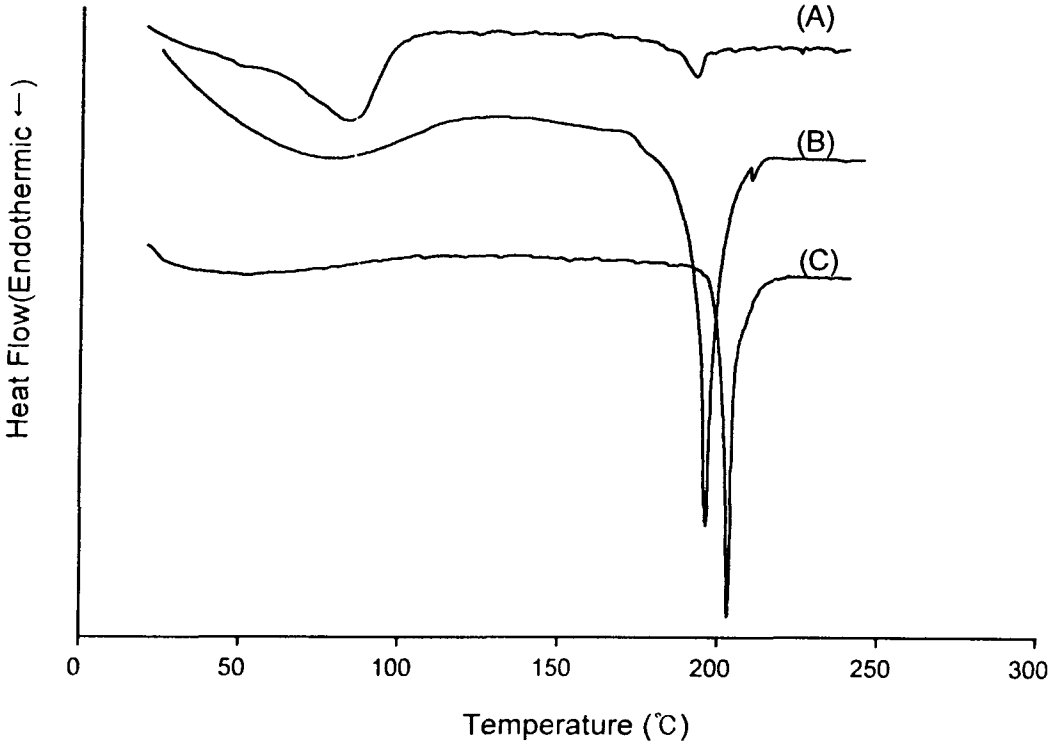

Figure 4. Differential thermal analysis of pure LG 106W(B), inclusion compound(C), and physical mixture(A).

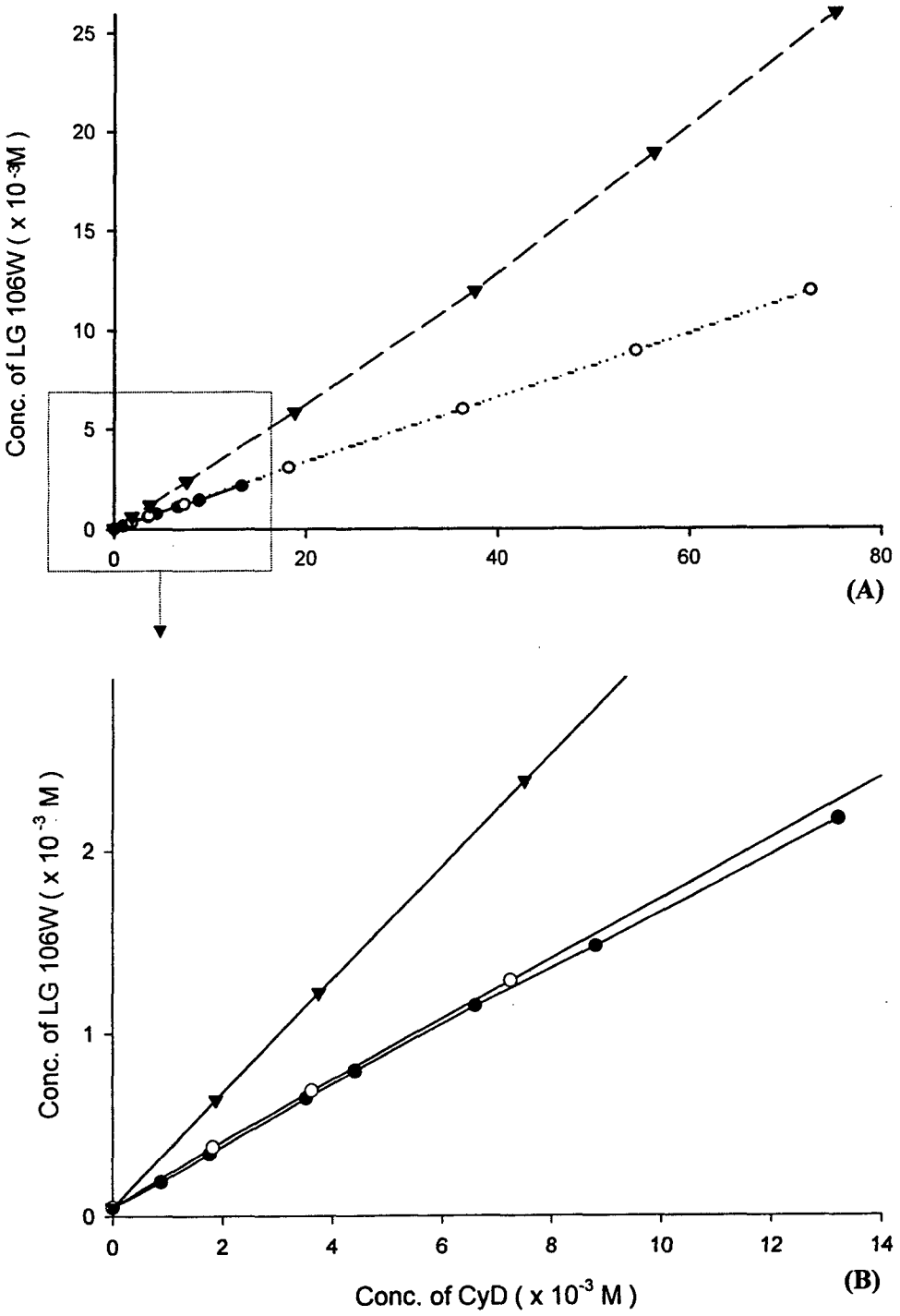

Figure 5. Phase solubility diagrams of LG 106W-CyD systems in 0.1M phosphate buffer. (●, β-CyD; ○, HP-β-CyD; ▼, DM-β-CyD)

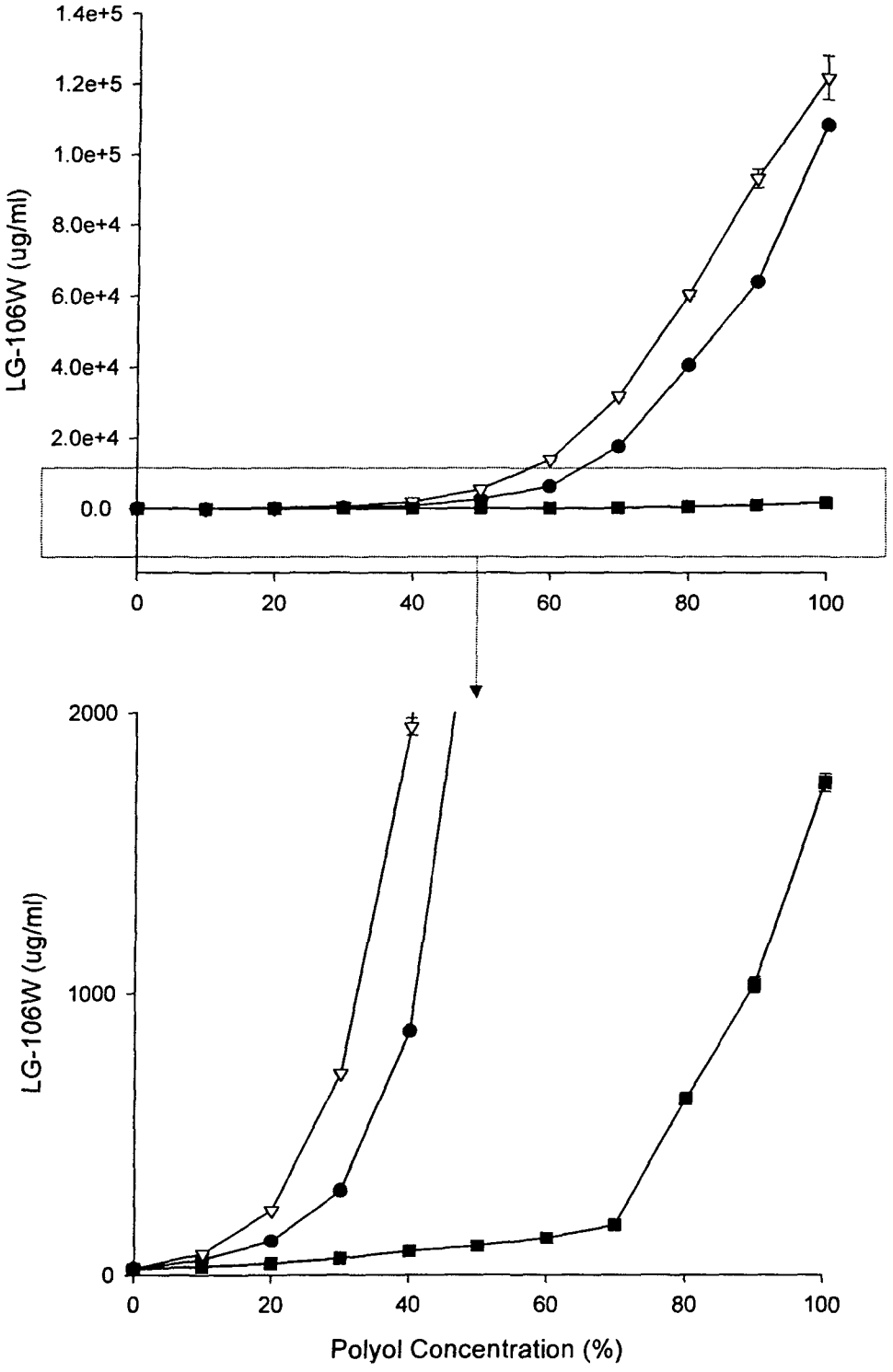

Figure 6. Polyol solubility diagrams of LG-106W at room temperature.
▽, 1,3-BG; ●, PG; ■, Glycerin

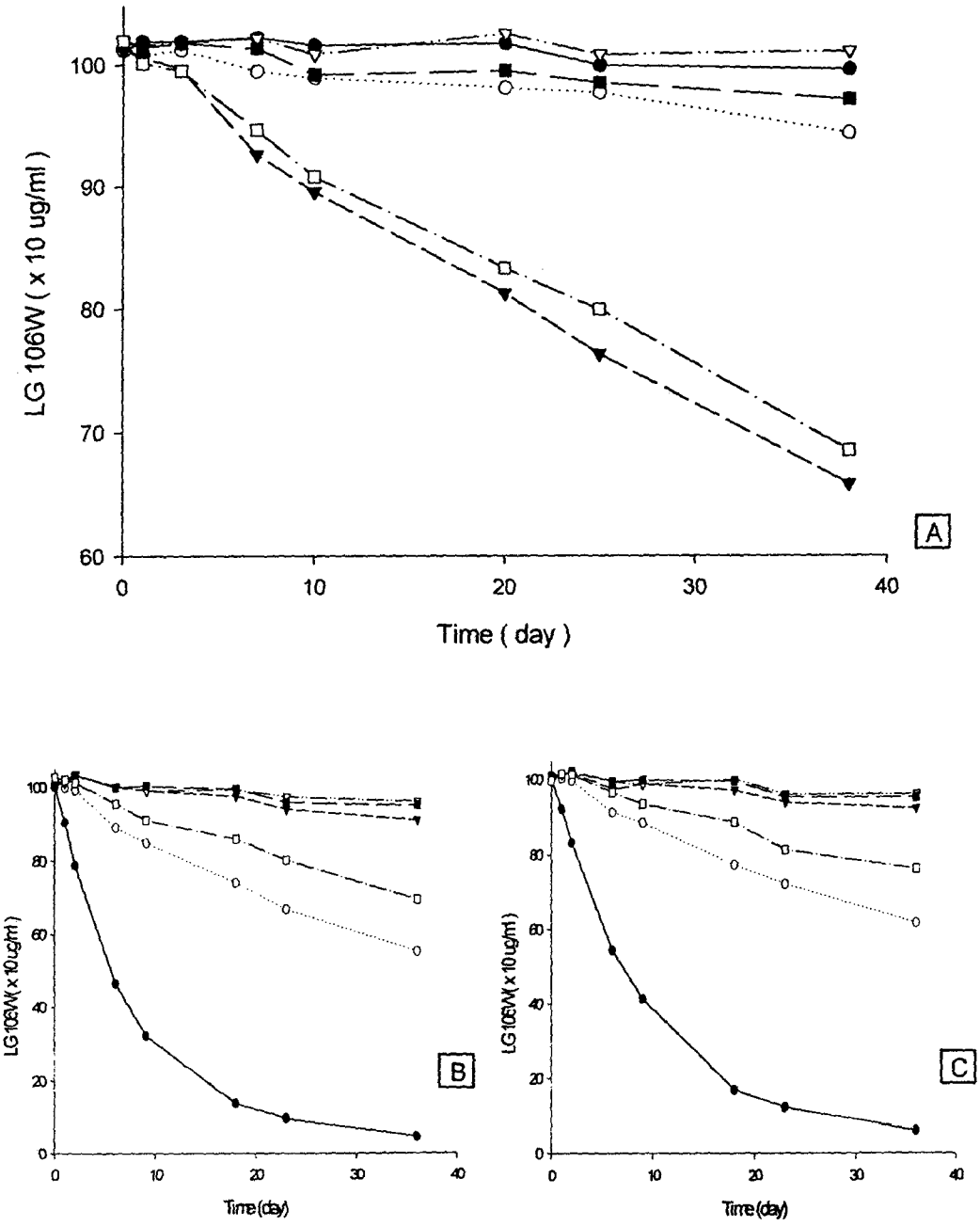

Figure 7. Stability comparison of LG 106W between free and complex(with HP-β-CyD) in 40% 1,3-BG solution. Temperature stability : [A], [free ●, 25℃; ○, 40℃; ▼, 50℃], [complex ▽, 25℃; ■, 40℃; □, 50℃], pH stability: free [B] , complex [C] , ●, pH 3.6; ○, pH 4.6; ▼, pH5.6; ▽, pH 6.7; ■, pH 7.7; □, pH 8.7

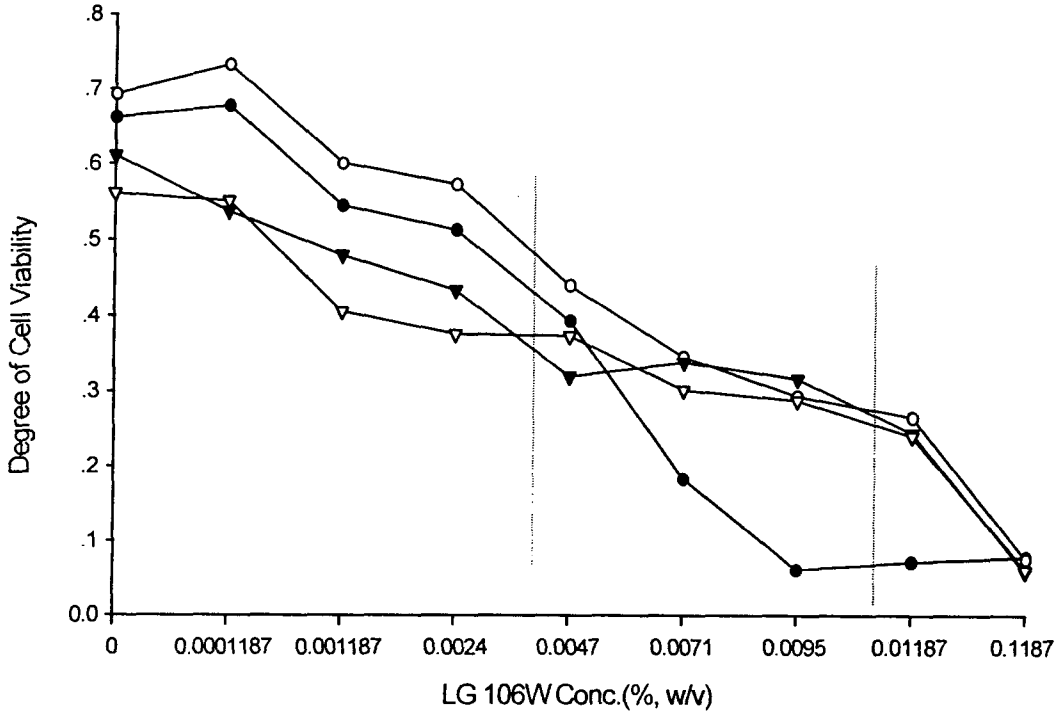

Figure 8. In vitro cytotoxicity test.
●, LG 106W; ▽, complex(5%); ○, complex(1:1); ▼, complex(1:2)