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Formation of Natamycin:Cyclodextrin Inclusion Complexes and Their Characterization

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Natamycin is a broad spectrum antimycotic with very low water solubility, which is used to extend the shelf life of shredded cheese products. β-Cyclodextrin (β-CD), hydroxypropyl β-cyclodextrin (HP β-CD), and γ-cyclodextrin (γ-CD) were found to form inclusion complexes with natamycin in aqueous solution. The increase in solubility of natamycin with added β-CD was observed to be linear (type A_L phase solubility diagram). The 1:1 stability constant of natamycin:β-CD complex was estimated from its phase solubility diagram to be 1010 M^{-1}. The phase solubility diagrams of both γ-CD and HP β-CD exhibited negative deviation from linearity (type A_N diagram) and, therefore, did not allow the estimation of binding constants. The water solubility of natamycin was increased 16-fold, 73-fold, and 152-fold with β-CD, γ-CD, and HP β-CD, respectively. The natamycin:CD inclusion complexes resulted in in vitro antifungal activity nearly equivalent to that of natamycin in its free state.

KEYWORDS: Natamycin; pimaricin; polyene macrolide; antifungal; preservative; cyclodextrin; inclusion complex; solubility

INTRODUCTION

Natamycin is a polyene macrolide antibiotic that is used as an antifungal agent in food preservation. In the U.S., natamycin may be applied on cheese in amounts not to exceed 20 ppm in the finished product and is no longer restricted in its method of application, which may include dipping, spraying, or adding as a dry mixture with safe and suitable anticaking agents (*1*). In shredded cheese products, mold spoilage is commonly attributed to the increased surface area of the cheese shreds and the extra handling and exposure that the shreds experience in the cut and packing facility. Natamycin possesses a much broader spectrum of activity than any other fungicide allowed for food application (*2*, *3*).

The very low aqueous solubility (30−50 mg/L) of natamycin (*4*−*6*) does not allow the preparation of a concentrated stock solution. Therefore, it is applied as an aqueous suspension to the shredded cheese surface, which results in clogging of spray nozzles and a heterogeneous distribution to the cheese surface. Solubility may be a limiting factor in the bioavailability of active natamycin, since the dissolved fraction must diffuse to the site of action and bind to the target organism (*7*). The application of natamycin as a dry powder mixed with an anticaking agent, such as cellulose, also likely limits the availability of natamycin to elicit an antifungal response.

Natamycin is more soluble in highly alkaline and acidic aqueous solutions, but the compound is rapidly degraded under such conditions (*6*). The ability to increase the aqueous solubility of natamycin by cholate formation, similar to the solubilization of amphotericin B by sodium deoxycholate, provided very limited success. SS-Natamycin was a water-soluble complex of approximately 66% natamycin and 34% of a modified polysaccharide but exhibited decreased biological activity and increased toxicity (*8*). The synthesis of a natamycin derivative that was truly water-soluble, *N*-(3′-*N*-dimethylaminopropylsuccimido)natamycin, was found to have decreased biological activity (*9*). It is possible that the original structure of natamycin (**Figure 1**) may be optimal and that any chemical modification decreases its activity.

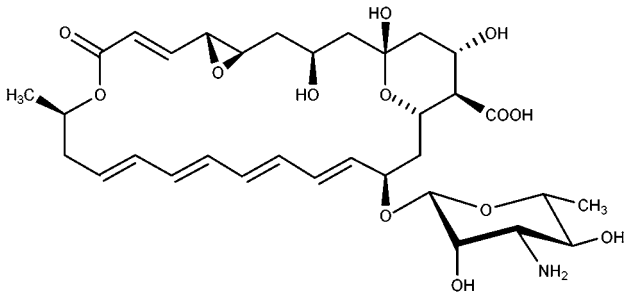

Figure 1. Structure of natamycin.

Cyclodextrins (CDs) act as host molecules to form inclusion complexes rather nonspecifically with a wide variety of guest molecules. The only apparent requirement is that the guest molecule must fit into the cavity, even if only partially (*10*). Complexation of guest compounds with CDs can alter guest solubility, increase stability against the effects of light, heat, and oxidation, mask unwanted physiological effects, and reduce volatility (*11*). The most common application of CDs in the

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pharmaceutical industry is to enhance drug solubility in aqueous solutions. In general, the lower the aqueous solubility of the pure drug, the greater the relative solubility enhancement gained by CD complexation (*12*). Other polyene macrolide antibiotics, including amphotericin B (*13*, *14*), nystatin (*15*), and flavofungin (*16*), have been complexed with γ-CD, resulting in greater than 40-fold increases in water solubility.

Cyclodextrin complexation offered the possibility to improve the aqueous solubility of natamycin without modification of its original structure. This may allow a homogeneous delivery system of natamycin to the shredded cheese surface that also increases its bioavailability. Although natamycin is a rather bulky molecule, all three of the native CD cavity sizes were examined, since inclusion complexation may only require a portion of the guest molecule to enter the cavity.

MATERIALS AND METHODS

Materials. Natamycin of 90.5% purity was supplied by DSM Food Specialties (Delft, The Netherlands). α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD; food grade); hydroxypropyl β-cyclodextrin (HP β-CD; pharmaceutical grade (5.3−5.4 DS)), and γ-cyclodextrin (γ-CD) were provided by Cerestar (Hammond, IN). High-purity water was prepared with a Mega-Pure System MP-6A (Corning Inc., Corning, NY) unless specified otherwise.

UV Absorption Spectrophotometry. Differential spectrophotometry was performed with a Shimadzu UV-2101PC UV−vis scanning spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) to quantify pure natamycin and natamycin content in its CD inclusion complexes in aqueous solution. A standard curve of natamycin in water was prepared, accounting for the sample purity of natamycin in its trihydrate form. The use of such a standard curve to determine the natamycin concentration in each CD complex involves the assumption that complexation does not substantially alter the molar absorption coefficient. The average absorbance of the minima at 296 and 312 nm was subtracted from the absorbance maximum at 304 nm to quantify the natamycin concentration present in aqueous solution. Complete spectrophotometric scans between 280 and 340 nm were monitored to detect any spectral shifts when natamycin was complexed with each CD type.

Solubility Equilibrium of Complexes. Several time points were selected with decreasing frequency until a duration of 168 h to monitor when solubility equilibrium was achieved. Aqueous solutions of 16 mM β-CD, HP β-CD, and γ-CD were prepared in duplicate in amber glass vials. This equimolar concentration was chosen, since it is the greatest solubility of β-CD that may be prepared in water at ambient temperature. Natamycin was added in great excess of its intrinsic solubility at concentrations of 2.0 g/L to each of the CD solutions. Each of these suspensions was ultrasonicated in a VWR Aquasonic 250D water bath (VWR Scientific Products, West Chester, PA) in the frequency range 38.5−40.5 kHz for 5 min to increase complexation efficiency. These suspensions were mechanically shaken in an Orbit Environ-Shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at 200 rpm for several time intervals. At each time point, the sample was filtered through a 0.45 μm membrane and appropriately diluted for quantitative analysis by UV differential spectrophotometry.

Phase Solubility Diagrams. Phase solubility diagrams were constructed to determine the apparent solubility increase of natamycin in the presence of each CD type. Amber glass vials containing aqueous solutions of α-CD, β-CD, HP β-CD, and γ-CD were prepared in triplicate with increasing concentration levels. CD concentration was varied in the range of 0−190 mM for both HP β-CD and γ-CD. The lower water solubilities of α-CD and β-CD only allowed a concentration range of 0−130 and 0−16 mM, respectively. Natamycin was then added to these vials in great excess of its intrinsic solubility at concentrations of 5.0, 2.0, 6.0, and 5.0 g/L for α-CD, β-CD, HP β-CD, and γ-CD, respectively. Each of these suspensions was ultrasonicated for 5 min, as described previously. These suspensions were mechanically shaken in an Orbit Environ-Shaker at 200 rpm at ambient temperature for 24 h to achieve equilibrium. Each sample suspension was filtered through a 0.45 μm membrane, and the filtrate was appropriately diluted for quantitative analysis of natamycin in each CD complex by UV differential spectrophotometry.

Natamycin in the absence of any CD was used to determine the intrinsic solubility of natamycin in water with 12 replicate samples. Aqueous solutions of HP β-CD (50.0% w/v) were also prepared in triplicate, to which an excess natamycin concentration of 10 g/L was added. The same method as above was then utilized. These data points were not included in the construction of the phase solubility diagram of HP β-CD, only in the evaluation of the apparent solubility increase.

Preparation of Solid Complexes. Solid inclusion complexes of natamycin with β-CD, HP β-CD, and γ-CD were obtained by complexation in aqueous solution. Solutions of 16 mM β-CD, 70 mM HP β-CD, and 70 mM γ-CD were prepared in HPLC water. These concentrations were chosen for their relatively high complexation efficiency based on their respective phase solubility diagrams. Natamycin was then added in great excess of its intrinsic solubility at concentrations of 2.0, 6.0, and 5.0 g/L to the β-CD, HP β-CD, and γ-CD solutions, respectively. Each of these suspensions was then ultrasonicated for 5 min, as described previously. These suspensions were protected from light by wrapping them with aluminum foil and stirred rapidly for 48 h to achieve solubility equilibrium. Each suspension batch was filtered through a 0.45 μm nylon membrane to obtain clear aqueous solutions of natamycin and each CD type. The sample filtrate was quantified by UV differential spectrophotometry. These solutions were then lyophilized in a laboratory freeze-dryer (Virtis, Gardiner, NY). The resulting samples were very flaky powders of low density. An electronic shaker (Janke & Kunkel, Staufen, Germany) was used on a high setting to compact the sample powders.

In vitro Antifungal Activity. Minimum inhibitory concentration (MIC) studies for natamycin and each of its CD complexes (β, HP β, and γ) were performed simultaneously for *Saccharomyces cerevisiae* and *Candida albicans*. *S. cerevisiae* Hansen (ATCC 9763) and *C. albicans* Berkhout (ATCC 10231) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Yeasts were cultured in Sabouraud Liquid Medium (Oxoid, Ogensburg, NY) and then diluted with physiological saline (2% NaCl w/v) to obtain a concentration of 10^4 CFU/mL prior to inoculation. Malt Extract Agar (Difco, Detroit, MI) was prepared in Petri dishes with natamycin and the natamycin content of its CD complexes was set at concentrations of 25, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 μg/mL. Natamycin stock solution was prepared in methanol at a concentration of 1000 μg/mL, since it is practically insoluble in water. Stock solutions of natamycin complexes with β-CD, HP β-CD, and γ-CD were prepared in water with 500, 1000, and 1000 μg/mL natamycin content, respectively. The concentration of natamycin present in each of the CD complexes was confirmed by UV differential spectrophotometry. Petri dishes were stored overnight in the dark in order to allow natamycin and its CD complexes to diffuse to completion in the media prior to inoculation. Malt Extract Agar plates were surface-inoculated with 100 μL yeast suspensions, described previously, and were incubated in the dark for 3 days at 25 °C. The lowest concentration at which no visible growth occurred at the end of the incubation period was determined to be the MIC value.

Particle Size Distribution. Laser diffraction particle size distribution analysis of the prepared natamycin:CD inclusion complexes was performed on a Horiba Model LA-700 particle size analyzer (Horiba Instruments Inc., Irvine, CA). Light mineral oil was used as the dispersion medium.

Thermogravimetric Analysis. A TGA 2950 thermobalance (TA Instruments, New Castle, DE) was used to differentiate between true inclusion complexes and physical mixtures of natamycin and CD. The TGA was calibrated with "alumel" alloy and nickel for temperature settings and with a 100 mg standard for weight accuracy. Approximately 10 ± 1 mg of sample was placed on a tared aluminum balance pan and transferred to the furnace at room temperature, where the exact sample weight was determined. The temperature program increased the temperature at a rate of 10 °C/min to 400 °C, and N_2 was used as the purge gas at a flow rate of 90 cm^3/min. Physical mixtures of natamycin and each CD type were prepared with natamycin contents (w/w) identical with those in its solid CD complexes. Samples of natamycin, CDs, their physical mixtures, and their inclusion complexes

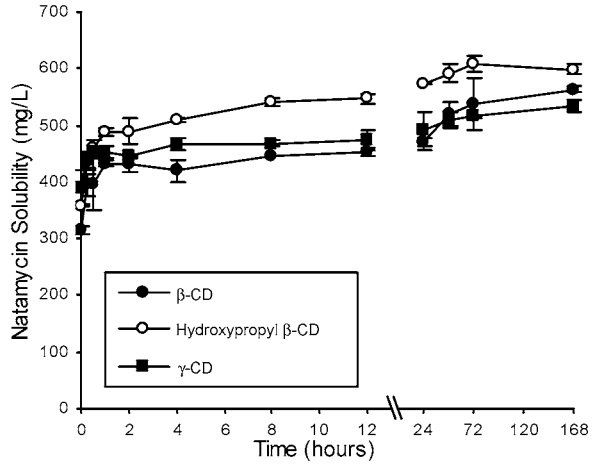

Figure 2. Equilibration time of natamycin to form cyclodextrin (CD) inclusion complexes with 16 mM solutions of β-CD, HP β-CD, and γ-CD in water at ambient temperature.

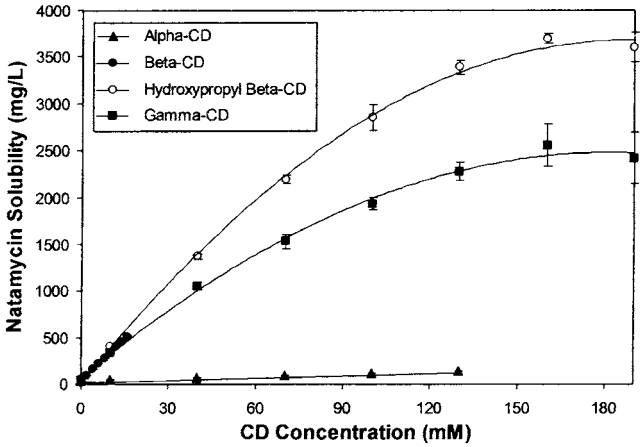

Figure 3. Water solubility of natamycin as a function of cyclodextrin (CD) concentration at ambient temperature for α-, β-, HP β-, and γ-CD.

were analyzed. Data were managed using TA Instruments Universal Analysis software, version 2.6D.

RESULTS AND DISCUSSION

Solubility Equilibrium of Complexes. The time required for solubility equilibrium of inclusion complexes to be achieved varies widely from the usual period of 1−2 days to durations of 1−2 weeks (*17*). The time necessary for natamycin to achieve solubility equilibration with β-CD, HP β-CD, and γ-CD follows a similar trend, which suggests that these complexes have binding constants of similar magnitude (**Figure 2**). The majority of natamycin complexation with each CD type occurs within the first 1 h, and after a period of 24 h the equilibration rate slows considerably; therefore, phase solubility analysis was conducted after 24 h of equilibration. Solid natamycin:CD complexes were prepared after 48 h of equilibration in order to increase the complexation efficiency even slightly further (**Figure 2**).

Phase Solubility Diagrams. In the absence of CD, the equilibrium water solubility of natamycin (s_0) was determined to be 34 ± 2.4 mg/L. Phase solubility diagrams were constructed by plotting the total concentration of dissolved natamycin (S_t) against the total CD concentration (L_t) for the four different CD types studied at ambient temperature (**Figure 3**). The solubility of natamycin shows a linear increase with unchanged stoichiometry as the β-CD concentration increases. According to Higuchi and Connors (*18*), a linear phase solubility diagram is classified as type A_L and the solubility limit of natamycin is determined by the solubility of β-CD. The solubility of natamycin in the presence of HP β-CD and γ-CD exhibits a negative deviation from linearity at relatively high concentration levels of CD. The negative curvature of these solubility diagrams is classified as type A_N, which infers an increase of the CD ratio within the complex, a change in the solute−solvent interaction, or a combination of both (*19*).

The solubility of natamycin only increased 4 times in the highest concentration of α-CD (12.6% w/v) used. This increase in solubility can be attributed to solvent effects and not the formation of a true inclusion complex. Natamycin solubility increased 16-fold, 105-fold, and 73-fold at the highest concentrations of β-CD (1.8% w/v), HP β-CD (27.1% w/v), and γ-CD (24.6% w/v) used during phase solubility analysis, respectively. A 50.0% w/v solution of HP β-CD allowed a 152-fold increase in natamycin solubility in water. After 2 weeks of ambient storage of these samples, the natamycin complexes of β-CD and HP β-CD were observed to have no precipitate formation. γ-CD concentrations of 130 mM or less which were complexed with natamycin did not have any precipitate.

Estimation of Binding Constant. A slope of less than 1 with a type A_L diagram does not necessarily indicate that only a 1:1 complex is formed, but this is a common assumption, which was made in the case of the natamycin:β-CD complex (*18*). A CD complex with a 1:1 stoichiometric ratio has the equilibrium

$$S + L \rightleftharpoons SL$$

The stability constant (K_{11}) for this equilibrium is defined as

$$K_{11} = \frac{[SL]}{[S][L]} \quad (1)$$

The 1:1 natamycin:β-CD complex has a stability constant that was calculated to be 1010 M^{-1}, using the equation

$$K_{11} = \frac{\text{slope}}{s_0(1 - \text{slope})} \quad (2)$$

where s_0 is the equilibrium solubility of natamycin in the absence of β-CD (*17*). The value of K_{11} cannot be calculated from the type A_N diagram (*19*), although estimates from the linear portion of the type A_N diagrams for HP β-CD and γ-CD indicate stability constants of the same order of magnitude.

Molecular Dispersion of Natamycin in Water. Solutions of polyene antibiotics have been reported to exist as micellar suspensions in aqueous media (*5*, *20*). The degree of dispersion of natamycin in water appears to be much greater than for amphotericin B, approaching closer to a molecular dispersion or true solution. Natamycin very precisely follows the Beer−Lambert law for its UV absorption spectra and has no apparent degradation of its strong vibrational fine structure in aqueous solution.

The heptaene amphotericin B has been shown to disobey the Beer−Lambert law on the basis of its circular dichroism and UV absorption spectra. These properties indicate that amphotericin B exists in a relatively labile aggregated form in aqueous solution. Light-scattering measurements in aqueous solutions of amphotericin B suggest an aggregated system of about 2000 molecules (*21*). The UV absorption spectra of amphotericin B in aqueous solution is highly degraded, which is in contrast to the strong vibrational fine structure of amphotericin B in a dispersive medium, such as 50% aqueous ethanol (*22*). Aggregation of molecules affects the free oscillation of electrons

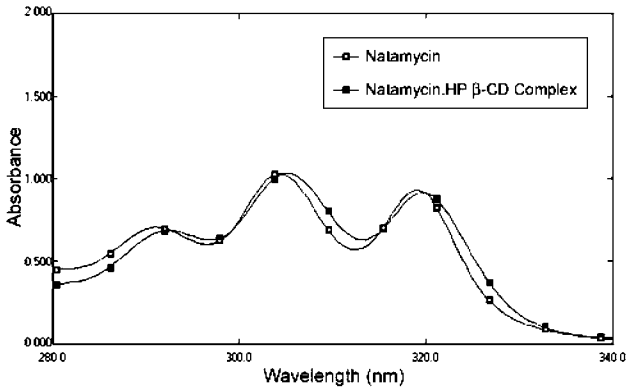

Figure 4. UV absorption spectrum of natamycin in water and the occurrence of a slight red shift and band broadening when natamycin is complexed with hydroxypropyl β-cyclodextrin (HP β-CD).

along the chain of the conjugated double bonds and results in spectral degradation, which is most severe in the fine structure (*4*).

Shift of UV Absorption Spectrum. The UV spectra of guests at adequately high CD concentrations are typically similar to those observed in ethanolic solutions. The UV spectrum of a concentrated aqueous solution of the amphotericin B:γ-CD complex was shown to be nearly identical with those observed in ethanolic solution (*23*). The presence of γ-CD strongly shifts the equilibrium toward the disaggregated form of amphotericin B. Complexation of natamycin with β-CD, HP β-CD, or γ-CD does not result in any considerable change in the shape of the strong vibrational fine structure in the UV absorbance spectrum of natamycin.

Formation of inclusion complexes with β-CD, HP β-CD, and γ-CD causes a slight red shift and band broadening of the UV absorption spectrum of natamycin (**Figure 4**). This effect may be explained by the high electron density existing inside the CD cavity, which partially shields the excitable electrons and chromophores of guests that are located within (*19*, *24*). Such a small, discrete spectral shift of about 1 nm appears to strongly indicate formation of a true inclusion complex. The UV absorption spectrum of natamycin did not exhibit any shift in the presence of α-CD, which provides further evidence that a true inclusion complex is not formed between natamycin and α-CD. Due to the very slight shift of the absorption spectrum of natamycin, any changes in the molar absorption coefficient were believed to be negligible.

In vitro Antifungal Activity. The biological activity of natamycin was compared to that of its CD complexes by performing MIC studies using the agar dilution technique. The improvements in aqueous solubility of natamycin overcome one of the disadvantages of this technique, which is poor solubility and distribution of the antibiotic in the agar medium (*25*). *Saccharomyces cerevisiae* ATCC 9763 is the standard fungal strain for the biological assay of natamycin (*26*), and *Candida albicans* is commonly used to determine the biological activity of polyene macrolides. Antifungal susceptibility testing in vitro is commonly characterized by a poor standardization and reproducibility of most of the methods employed (*27*, *28*). As a consequence, MIC experiments of each natamycin:CD complex were performed simultaneously with natamycin as the reference. The MIC value for natamycin is reported as the mean of these three values, and MIC values of each natamycin:CD complex are listed in **Table 1**. *S. cerevisiae* showed similar susceptibility to natamycin in its free and CD complexed states. *C. albicans* showed similar susceptibility to natamycin and its β-CD and HP β-CD complexes, while the natamycin:γ-CD complex displayed an increase of one MIC concentration level. Thus, the antifungal activity of the CD complexes appears to be nearly equivalent to that of the parent compound.

Table 1. In Vitro Antifungal Activity of Natamycin and Its Cyclodextrin (CD) Inclusion Complexes

	min inhibitory concn (μg/mL)			
organism	natamycin (mean, $n = 3$)	natamycin: β-CD complex	natamycin: HP β-CD complex	natamycin: γ-CD complex
Candida albicans (ATCC 10231)	2.6 ± 0.9	1.6	1.6	6.3
Saccharomyces cerevisiae (ATCC 9763)	1.1 ± 0.5	0.8	0.8	1.6

Table 2. Mean and Median Particle Size of Natamycin, Cyclodextrins (CDs), and Their Inclusion Complexes

	particle size (μm)	
compd	mean	median
natamycin	19.88	12.36
β-CD	50.62	30.24
hydroxypropyl β-CD	72.87	50.77
γ-CD	71.09	66.94
natamycin:β-CD complex	39.07	28.51
natamycin:hydroxypropyl β-CD complex	69.37	58.43
natamycin:γ-CD complex	67.51	48.03

Complexes as Solid Dry Powders. The efficiency of complexation may sometimes be rather low, and therefore, relatively large amounts of CDs must be used to complex small amounts of drug. Typically, solid drug−CD complexes contain less than 5−10% of the drug (*12*, *29*). The antibiotic content (w/w) of solid γ-CD complexes was between 7 and 8% for nystatin, 0.6−0.7% for amphotericin B, and 9−10% for flavofungin (*16*). The natamycin content (w/w) in its CD complexes as a solid dry powder was 2.8% for β-CD, 2.4% for HP β-CD, and 2.1% for γ-CD.

The particle size of solid CD complexes can affect the dissolution rate and, therefore, the bioavailability of the product. The particle size distribution and crystalline properties of the complex are dependent on the method of complex preparation (*30*). Complexation in aqueous solution followed by freeze-drying to obtain each natamycin:CD complex in solid form results in an increase in particle size relative to pure natamycin (**Table 2**). The median particle size increases approximately 2 times for the β-CD complex, 5 times for the HP β-CD complex, and 4 times for the γ-CD complex.

Thermogravimetric Analysis. Thermal analysis has mainly been applied to demonstrate the different behavior of an inclusion compound relative to its physical mixture of component compounds (*24*, *31*). Most of the water molecules within the CD cavity and in the trihydrate form of natamycin are released by a temperature of 100 °C. The initial decomposition temperature of natamycin at 198.2 °C only results in a 16% weight loss (**Figure 5**). Only an approximately 0.4% decrease in weight would be observed upon decomposition of natamycin in either the CD complexes or physical mixtures, since the natamycin content of the solid CD complexes is only about 2.5% (w/w) and there is not a complete degradation of the guest at 198.2 °C. Therefore, it was beyond the sensitivity of the method to demonstrate different behaviors between the inclusion complexes and the physical mixtures. The TGA thermograms

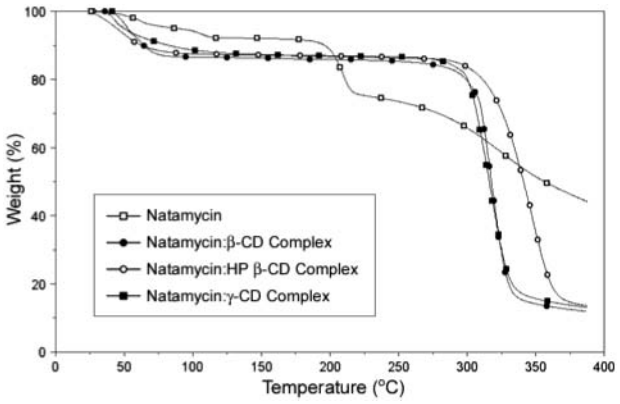

Figure 5. TGA thermograms of natamycin and its cyclodextrin (CD) inclusion complexes for β-, HP β-, and γ-CD under N_2 at a temperature rate of 10 °C/min.

of each type of natamycin:CD complex appear nearly identical with the thermograms of their respective CD in pure form.

Improved Properties of Natamycin. The formation of cyclodextrin inclusion complexes with natamycin has allowed large improvements in the aqueous solubility of natamycin without modification of its original structure or antifungal activity. The increases in aqueous solubility allow a homogeneous distribution of natamycin to be applied to the shredded cheese surface without the clogging of spray nozzles during cheese production.

ABBREVIATIONS USED

CD, cyclodextrin; α-CD, α-cyclodextrin; β-CD, β-cyclodextrin; HP β-CD, hydroxypropyl β-cyclodextrin; γ-CD, γ-cyclodextrin; DS, degree of substitution.

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