The Application of Crystal Soaking Technique to Study the Effect of Zinc and Cresol on Insulinotropin Crystals Grown from a Saline Solution

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Received February 27, 1995; accepted June 14, 1995

Purpose. The purpose of this study is to investigate the effect of zinc and cresol on the structure of insulinotropin crystals.

Methods. Insulinotropin crystals grown from a saline solution were treated with zinc and/or m-cresol using a crystal soaking technique. The effects of these additives on the crystal structure were investigated with powder X-ray diffraction, photomicrography, and differential scanning calorimetry. The molecular interaction between insulinotropin and m-trifluorocresol in solution was also studied by ^{19}F NMR.

Results. The data suggest that the original crystals grown from a saline solution have relatively weak lattice forces. After the addition of m-cresol to the suspension of the insulinotropin crystals, the crystals were immediately rendered amorphous. The m-cresol molecules which diffused into the crystals through solvent channels may have disturbed the lattice interactions that maintain the integrity of the crystal. In contrast, the zinc added to the suspension stabilized the crystal lattice so that the subsequent addition of m-cresol did not alter the integrity of the crystals. A marked increase in melting point (206° versus 184°) and heat of fusion (24.6 J/g versus 1.4 J/g) of the crystals was observed after the treatment with zinc. The solubility of the zinc treated crystals in a pH 7.1 phosphate buffered saline was 1/20 of that of the original crystals.

Conclusion. When the insulinotropin crystals were treated with the additives using a crystal soaking method, the crystals underwent structural changes. Zinc stabilized the crystal lattice, and reduced the solubility of the peptide.

KEY WORDS: glucagon-like-peptide-I-(7-37); crystal; solubility; zinc; m-cresol.

INTRODUCTION

Insulinotropin (Glucagon-like-peptide-I-(7-37)) is a peptide containing 31 amino acid residues (1,2). This peptide is 100 times more potent than glucagon in stimulation of insulin secretion, and insulin release in response to insulinotropin is highly dependent on the ambient glucose concentration (1,2). Therefore, it is thought that insulinotropin could be an important tool in the treatment of non-insulin-dependent diabetes mellitus. The half life of insulinotropin in humans is less than 5 min (3). Since prolonging the action of insulinotropin is expected to have benefits, the development of sustained release preparations for subcutaneous injection is desirable. One possible approach to achieve this goal would be crystallizing insulinotropin as a metal complex to reduce its solubility (4).

Two most common ways of making complexes of macro-molecules are co-crystallizing a complex directly from a solution which contains both macromolecules and a ligand (4–6), and soaking pre-formed crystals of the macromolecule in a ligand or reactant solution. In the process of solving protein crystal structures, the latter technique is frequently used to prepare heavy atom isomorphous derivatives of the macromolecule (7,8). The crystal soaking technique is also widely used to study enzyme-substrate binding (9,10). Danley et al. have performed a crystal soaking experiment with zinc solution and insulinotropin crystals grown from a buffer solution containing polyethylene glycol to prepare a zinc/insulinotropin complex (11). Subsequently, Chrunyk et al (12) observed an increase in far-UV circular dichroism of insulinotropin in the presence of zinc, which suggested that zinc stabilized the secondary structure of insulinotropin in solution. A similar phenomenon observed for insulin has been reported by Frank et al. (13).

Zinc ion is known to have some intrinsic affinity to the imidazole rings of histidine residue (14). An ^{1}H NMR study done by Chrunyk et al (12) also suggests the presence of a zinc binding site at the N-terminus of insulinotropin (for the sequence of the peptide, see Material section). The stoichiometry of zinc binding to insulinotropin had been determined by Chrunyk (15) using a method which was based on a calorimetric reaction between zinc and the reagent zincon (2-Carboxy-2′-hydroxy-5′-sulfoformazyl-benzene, sodium salt) (16). Zn/insulinotropin molar ratios between 0 and 5 were tested, and there was a consistent finding of an approximately 1 to 1 binding of zinc to insulinotropin (15).

Phenol and cresol are widely used as preservatives to control microbial growth for multi-use pharmaceutical and cosmetic products (17). We have observed that when phenol (18) or cresol was added to an insulinotropin solution prepared with pH 7 phosphate buffered saline, the peptide instantaneously precipitated out of solution. In our previous study, the secondary structure of insulinotropin precipitates that were prepared under different conditions was investigated in the solid state by FT-IR and Raman spectroscopy (18). For the insulinotropin that was precipitated upon the addition of phenol, a significant increase in the content of β-sheet was found at the expense of α-helix when compared with the original peptide (18).

In the present study, we investigated the effect of m-cresol and zinc on the physicochemical properties of insulinotropin crystals grown from a saline solution by using crystal soaking technique. It was found that these additives change the crystal structure and lattice forces of the peptide, which leads to alterations in its solubility and pharmacokinetics after subcutaneous injection.

MATERIALS AND METHODS

Material

The insulinotropin (molecular weight 3,338) used throughout this study was synthesized by Bachem California under contract to Pfizer. It was prepared by solid state peptide synthesis. The primary sequence of the peptide is ^{1}His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu- Phe-Ile-Ala-Trp-Leu-Val-

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0724-8741/95/1100-1664$07.50/0

Lys-Gly-Arg-^{31}Gly. Deionized water which was further purified by a Milli-Q water system (Millipore Corp., Bedford, MA) was used, α,α,α-Trifluoro-m-cresol (99%) and fluorotrichloromethane (NMR grade) were purchased from Aldrich. Dulbecco's phosphate buffered saline (PBS) was purchased from Gibco (Life Technologies, Inc.). All other chemicals were of reagent grade.

Preparation of Insulinotropin Suspensions

Preparation of insulinotropin suspensions is shown schematically in Figure 1. After insulinotropin was dispersed in 1% (w/v) NaCl, the pH was adjusted to 9.3 ± 0.2 with 1N-NaOH to obtain a clear solution. Then 0.1N-HCl was used to re-adjust the pH of the solution to pH 6.5 ± 0.1 which is close to the isoelectric point of insulinotropin (p*I* 5.3) (19). This solution (1.3 mM) was filtered through a 0.22 μm Millipore Millex-GV filter to remove dust and was allowed to sit at ambient temperature. Needle shaped or thin blade-like crystals started forming within 5 to 6 hours, and crystallization was complete within 2 days. This suspension of insulinotropin crystals was labeled as I-A.

A zinc stock solution (18 mM) was prepared by dissolving zinc acetate dihydrate in water (other zinc salts such as zinc chloride can be used). A homogeneous dispersion of m-cresol in water (230 mM) was prepared by sonicating the mixture of m-cresol and water. Suspension IZ-B was prepared by adding 10 ml of the zinc stock solution to 90 ml of suspension I-A, and aging overnight. Suspension IC-C was prepared by adding 10 ml of the m-cresol dispersion to 90 ml of suspension I-A stirring gently until the m-cresol dissolved completely. Suspension IZC-D was prepared by adding 10 ml of the m-cresol dispersion to 90 ml of suspension IZ-B stirring gently until the m-cresol dissolved completely and aging for one day.

The final pH of the suspensions I-A, IZ-B, IC-C, and IZC-D was adjusted to pH 7.2 ± 0.1 with 0.2N-NaOH before being used for the experiments described below.

Photomicrography

The birefringence of the crystals was examined under a polarized light microscope (Olympus BH-2) with crossed polarisers. The interaction between insulinotropin crystals and m-cresol was observed by positioning a cover slip on a microscope slide, adding small drops of suspension I-A and a 10 mg/ml solution of m-cresol in water to opposite sides of the cover slip, and allowing the drops to move towards the center of the cover slip to form a mixing zone. Photographs were taken at the mixing zone.

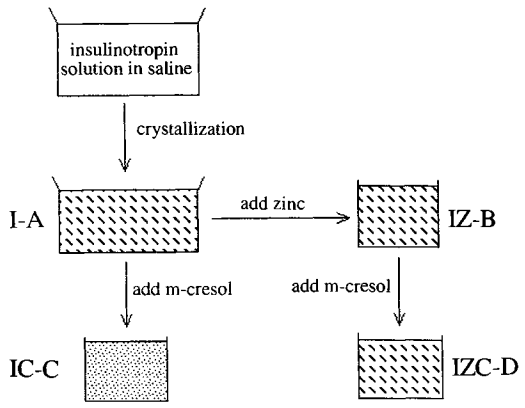

Fig. 1. Schematic representation of sample preparation for insulinotropin suspensions.

Powder X-Ray Diffraction

The powder X-ray diffraction patterns were measured using a Siemens D5000 powder diffractometer equipped with a Kevex detector. Samples of the suspensions were centrifuged, and the isolated solids were placed in a small circle quartz holder. The spectra were recorded using a θ/2θ coupled step scan method employing CuK_α radiation, a scan width of 0.01 degrees, a step time of 10.0 seconds, and a scan range from 1.5 to 30.0 degrees in 2θ. The Siemens D5000 employes a Bragg-Brentano focusing geometry (20), an incident beam slit of 1 mm, and detector slits of 0.6 and 1 mm.

^{19}F NMR Spectroscopy

^{19}F NMR spectra were recorded on a 300 MHz Bruker AC series NMR spectrometer. Samples were prepared in D_2O, and the pD of the samples was adjusted to 9.5 with NaOD to prevent insulinotropin from precipitating. Fluorotrichloromethane ($FCCl_3$) was used as a reference standard for chemical shift.

Differential Scanning Calorimetry (DSC)

Suspensions I-A and IZ-B were centrifuged at 14,000 rpm for 20 min. After removing the supernatant, the remaining solids were dried in a desiccator at room temperature for 2 days.

Scans of the samples were recorded on a Mettler DSC25 differential scanning calorimeter. Using Mettler Graph Ware TA72.2/.5, the instrument was able to calculate extrapolated onset temperature, peak temperature, and enthalpy values for each thermal event.

Bound Zinc and m-Cresol Assay

Additives bound to insulinotropin solids were determined for unwashed and washed samples. The zinc/insulinotropin molar ratio and m-cresol/insulinotropin molar ratio were calculated for the suspensions IZ-B and IZC-D and for the suspensions IC-C and IZC-D, respectively.

For the unwashed samples, each suspension was centrifuged to pellet the suspension solids. After removing the supernatant completely, the solid pellet was dissolved in 1% trifluoroacetic acid (TFA) in water. As appropriate, insulinotropin, zinc and m-cresol concentrations were then assayed by the methods described below.

For the washed samples, each suspension was centrifuged to pellet the solids. After removing the supernatant from the centrifuged sample, the solids were washed by adding a sufficient amount of water to the centrifuge tube to resuspend the pellet with vortex mixing. The suspension was centrifuged again to remove the supernatant. This procedure was repeated 2 or 6 times. The washed sample was dissolved in 1% TFA and was assayed for insulinotropin, zinc and m-cresol content as appropriate.

Zinc content was determined by Inductive Coupled Plasma (ICP) optical emission spectroscopy using an ICP Quantometer (Model 137100, Applied Research Laboratories, Dearborn, Michigan).

Insulinotropin and m-cresol assays were conducted with a Vydac Protein C4 column (Rainin Cat# 214TP54) and the following gradient program, where *A* represents 0.1% TFA in water and *B* represents 0.1% TFA in acetonitrile.

Gradient Program

Time (min)	0	5	30	35	37	46
%A	75	75	50	50	75	75
%B	25	25	50	50	25	25

The flow rate was 1 ml/min and the column was kept at ambient temperature. Detection was by UV absorption at 215 nm. Insulinotropin and m-cresol retention times were 23 min and 6.5 min, respectively. The equipment used for the HPLC assay included an LDC Consta-Metric 4100 solvent delivery system, a Bio-Rad Model AS-100 HRLC automatic sampling system, an LDC SpetcroMonitor 4100 programmable variable wavelength detector, and a SpectraPhysics Chrom Jet integrator.

Solubility Determination

The equilibrium solubility for the insulinotropin suspensions was determined at room temperature by dispersing an excess amount solids from the suspensions into PBS and vortexing. This dispersion was allowed to sit at ambient temperature for 24 hours. The dispersion was centrifuged to pellet the solids. The supernatant was filtered through a 0.22 micron Millipore Millex-GV filter and assayed by HPLC for insulinotropin concentration.

RESULTS

Morphology

The morphology of the suspension particles was examined under the microscope. Sample I-A was a mixture of needle shaped (or thin blade like) crystals and those grown from a central nucleation point as shown Figure 2A. Upon shaking, the latter can be easily dissociated into the former, whose length ranges from 8 to 15 μm. No difference in morphology was observed under the microscope among the samples I-A, IZ-B, and IZC-D, all of which exhibited strong birefringence under crossed polarisers (Figure 2B).

A dramatic change was visible immediately after the m-cresol addition to suspension I-A to form suspension IC-C. The particles became very fluffy and fine, and did not settle for days. Microscopy showed the particle diameter was less than 1 μm. When viewed under crossed polarisers, the particles were not birefringent, indicating they were amorphous. M-cresol concentrations ranging from 0.5 to 2.3 mM were tested, and all of them destroyed the crystals of suspension I-A.

Figure 2C is a 100 X magnification photograph of the crystals from suspension I-A before contact with the m-cresol solution. Figure 2D is a photograph taken at the moment when the insulinotropin crystals came into contact with the m-cresol solution at the mixing zone. The crystals were immediately rendered amorphous. When the same experiment was conducted with other small molecules such as phenol, o-cresol, p-cresol, p-nitrophenol, methylparaben, and toluene, all of them converted the crystals to an amorphous material. This extreme sensitivity to these aromatic compounds is indicative of a labile crystal structure, or an unidentified component that is critical for maintenance of the crystalline structure.

When the same mixing experiment was carried out under the microscope with the m-cresol solution and the crystals from suspension IZ-B, which had been treated with zinc in advance, the crystals remained intact. This suggests that the structure of the IZ-B crystals is more stable than that of the I-A crystals. Suspension IZC-D, which was prepared by addition of m-cresol to suspension IZ-B, was stored at 5, 25, and 50°C for 3 months. The morphology of the crystals remained the same at all the storage conditions.

Powder X-Ray Diffraction Pattern

Powder X-ray diffraction patterns of solids from the four suspensions were taken. As shown in Figure 3, sample IC-C did not show any diffraction pattern confirming that it is an amorphous material. On the other hand, samples I-A, IZ-B, and IZC-D showed distinct diffraction patterns. Table I lists 2θ values of diffraction maxima and *d* spacings derived from the Bragg equation (21)

$$\lambda = 2d\sin\theta$$

where λ is the wavelength of the radiation, *d* the spacing of the lattice planes of the crystal, and θ the angle at which the emergent ray is observed relative to the direction of the lattice planes.

Evidently, the *d* spacings of the crystals changed due to the treatments with zinc and m-cresol. For instance, each treatment appeared to enlarge the long spacing (25.48 Å for I-A → 26.27 Å for IZ-B → 27.23 Å for IZC-D) of the crystals. This indicates that insulinotropin crystals underwent structural changes due to the zinc, or zinc/m-cresol treatment.

^{19}F NMR Spectra

^{19}F NMR spectroscopy was used to study the interaction of m-trifluorocresol with insulinotropin by observing the chemical shift of the CF_3 singlet. The ^{19}F NMR spectra of m-trifluorocresol showed that the CF_3 singlet was broadened and shifted downfield by addition of insulinotropin (Figure 4). The line broadening, which usually occurs when a small molecule binds to a macromolecule (22), is interpreted as indicative of intimate contact between the fluorine atoms of m-trifluorocresol and the cresol binding site of the insulinotropin. It indicates m-trifluorocresol of rapid to intermediate exchange (relative to the NMR time scale) between the insulinotropin-bound environment and that in the solution (22,23).

DSC Scans

The DSC scans for samples I-A and IZ-B are shown in Figure 5. Hot stage experiments confirmed that the endotherms at 184.3°C for sample I-A and 206.3°C for sample IZ-B were melting events. The DSC scan for sample I-A

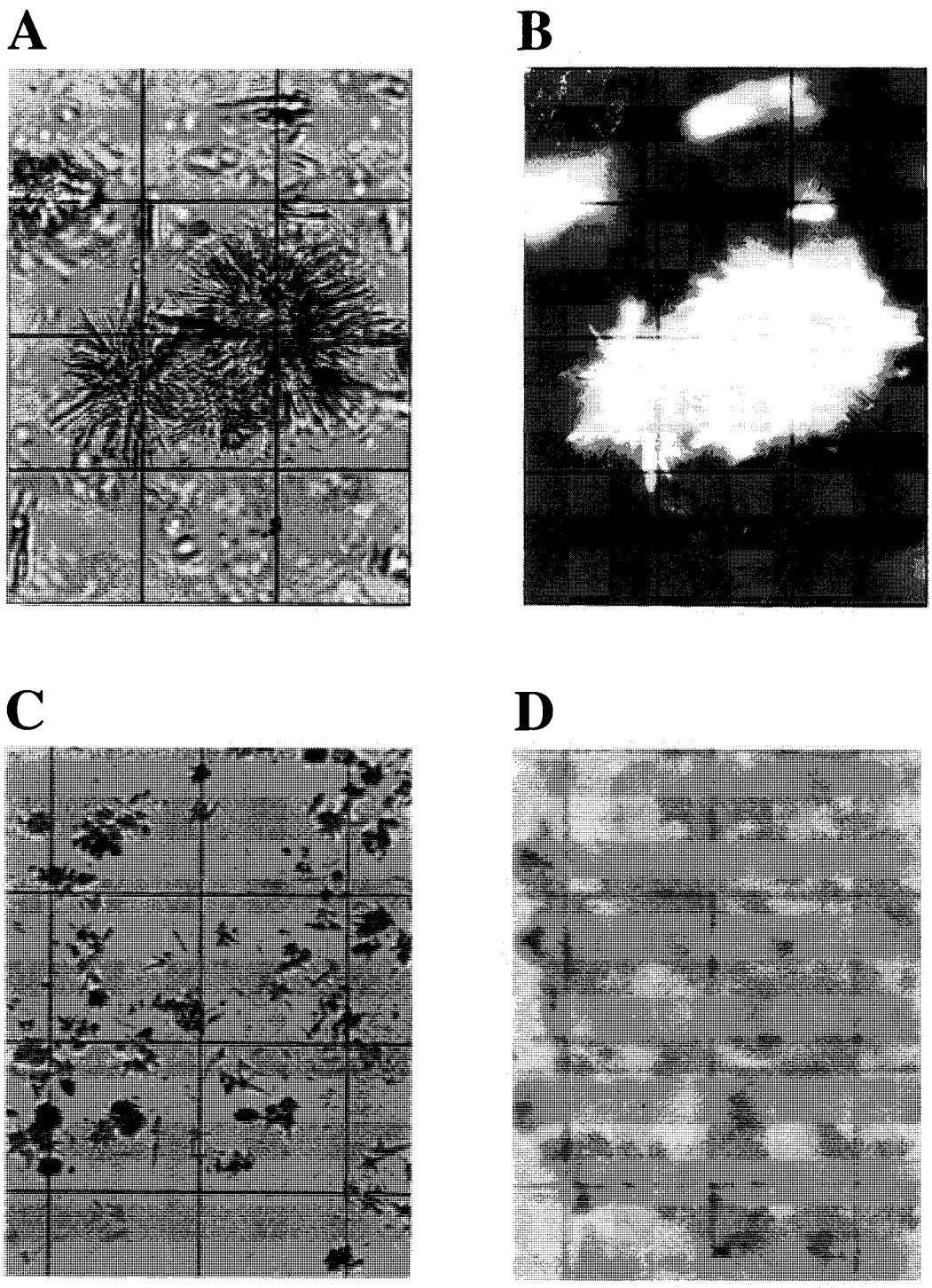

Fig. 2. Photographs of insulinotropin crystals grown from 1% (w/v) NaCl. The pictures (A), (B), and (C) were taken without a cover slip. Partially crossed polarisers and crossed polarisers were used for the pictures (A) and (B), respectively (grid size: 35 μm). The picture (D) was taken at the mixing zone of the crystals and m-cresol, and compared to the picture of the original crystals (C) which was taken at the same magification (grid size: 145 μm). For the detailed experimental conditions used for the picture (D), see text.

showed onset of melting at 164°C and a heat of fusion of 1.4 J/g. The DSC scan for sample IZ-B showed onset of melting at 190.6°C and a heat of fusion of 24.6 J/g. The heat of fusion of sample IZ-B could have been slightly overestimated, however, since this endotherm has a shoulder at about 215°C, which may be an indication of decomposition. Even when this is taken into account, both the onset and heat of fusion are significantly higher for sample IZ-B than for sample I-A. These data indicate that the crystal lattice was stabilized by the addition of zinc.

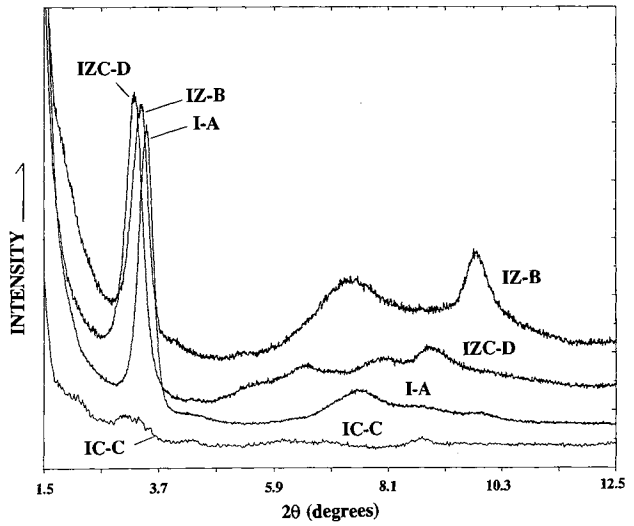

Fig. 3. Powder X-ray diffraction patterns of the isolated solids from suspensions I-A, IZ-B, IC-C, and IZC-D. For the detailed explanation of these samples, see text.

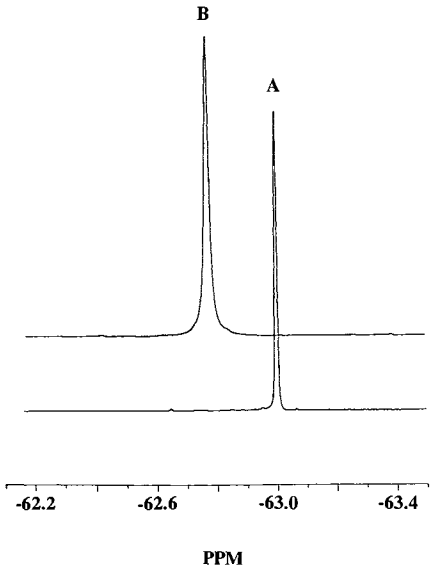

Fig. 4. ^{19}F NMR spectra of 1 mM m-trifluorocresol (A) and 1 mM m-trifluorocresol containing 1.5 mM insulinotropin (B). The positions of the CF_3 singlet resonance are $\delta(CF_3) = 63.011$ ppm for A and $\delta(CF_3) = 62.770$ ppm for B.

Bound Zinc and m-Cresol Concentrations

The zinc in the crystalline solids was assayed for the suspensions IZ-B and IZC-D as described above. The results are shown in Table II. The zinc/insulinotropin molar ratio was about one for the two unwashed samples, and did not change even after six washes. This indicates specific (i.e. high affinity) binding (24) of zinc to insulinotropin. This one to one molar ratio between zinc and insulinotropin is consistent with Chrunyk's finding (15).

As shown in Table II, the molar ratios of m-cresol/insulinotropin in the unwashed solids from suspensions IC-C and IZC-D were also close to one. In the case of the solids from suspension IC-C, the washing experiment could not be conducted due to their high solubility in water (see Table III). M-cresol concentration in the solid from suspension IZC-D decreased significantly after washing, and was not detectable after six washes (the detection limit was 0.4 μg/ml). This suggests nonspecific (i.e. low affinity) binding (20) of m-cresol to the peptide. Since there was no significant difference in m-cresol concentration between the unwashed solids from suspension IC-C and the unwashed solids from suspension IZC-D, zinc and m-cresol do not appear to compete for the same binding site. In the case of unwashed IZC-D, which contained 1.05 mM insulinotropin, the bound zinc was 1.02 mM and the free zinc was 0.6 mM. For the same sample, the bound m-cresol was 1.4 mM and the free m-cresol was 21.6 mM.

Table I. X-Ray Powder Diffraction Spacings of Insulinotropin Crystals of I-A, IZ-B, and IZC-D[a]

I-A		IZ-B		IZC-D	
2θ (°)	d (Å)	2θ (°)	d (Å)	2θ (°)	d (Å)
3.467	25.484(s,s)	3.363	26.272(s,s)	3.245	27.227(s,s)
				6.488	19.613(w,b)
7.532	11.727(w,b)	7.389	11.964(m,b)	7.976	11.076(w,b)
8.926	9.899(w,b)			8.913	9.922(w,b)
9.812	9.006(w,b)	9.771	9.052(m,s)		

[a] (s,s): strong and sharp, (w,b): weak and broad, (m,b): medium and broad, (m,s): medium and sharp.

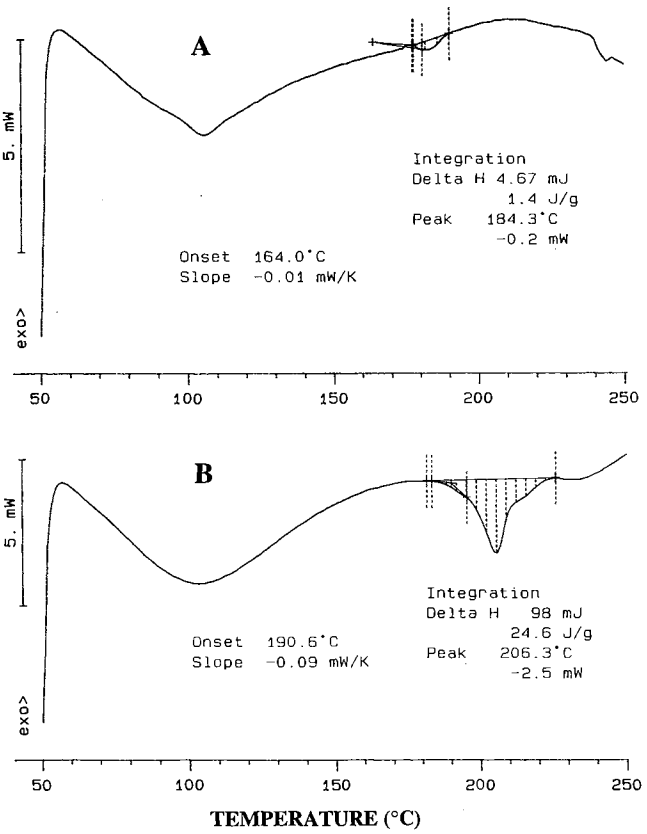

Fig. 5. Differential scanning calorimetry traces of the isolated solids from suspensions I-A (A) and IZ-B (B). The heating rate was 20°C/min. The sample sizes were 3.4 mg for I-A and 3.9 mg for IZ-B.

Table II. Zinc/Insulinotropin and m-Cresol/Insulinotropin Molar Ratios in the Washed and Unwashed Samples of IZ-B, IC-C, and IZC-D

Samples	Number of washing[a]	Molar ratios: Zn/ Insulinotropin	Molar ratios: m-Cresol/ insulinotropin
IZ-B	0	1.10	
IZ-B	2	0.96	
IZ-B	6	1.05	
IZC-D	0	0.97	1.33
IZC-D	2	1.03	0.08
IZC-D	6	1.10	<0.01[b]
IC-C	0		1.21

[a] centrifuge → remove the supernatant →
add water → vortex → centrifuge → remove the supernatant →
ONE WASHING

[b] m-Cresol was not detected by HPLC.

Solubility in a pH 7.1 Phosphate Buffered Saline (PBS)

The solubility of the solids from the suspensions in PBS is shown in Table III. The solids from suspension I-A showed a solubility of 0.45 mg/ml. Solubility of the solids from suspensions IZ-B and IZC-D was approximately equal (0.02 and 0.03 mg/ml, respectively), and much lower than that of solids from suspension I-A. Solubility of solids from suspension IC-C was 0.80 mg/ml which is much higher than that of solids from suspension I-A.

DISCUSSION

Binding of phenolic compounds to bovine carbonic anhydrase (25) and insulin hexamer (23,26) has been reported. The aromatic binding site of phenol (or similar molecules) is known to be a hydrophobic region of the macromolecules (25,26). Roy et al. (23) demonstrated, in their ^{19}F NMR study, line broadening of CF_3 singlet which was due to the binding of trifluorocresol to insulin hexamer.

The self-association of insulinotropin in solution (from 0.03 to 0.78 mM insulinotropin in 50 mM pH 9.0 Na-borate buffer) was characterized by Grucza et al. (27) using equilibrium analytical ultracentrifugation and circular dichroism. The study showed evidence that at the high protein concentration range studied, the peptide formed a tetramer species. Our ^{19}F NMR study suggests that there is a binding site for cresol in the insulinotropin molecule. For the study, we used an 1.5 mM insulinotropin solution, which was much higher than the highest concentration used by Grucza et al. Therefore, it is likely that insulinotropin formed at least tetramers in the solution. This higher-ordered, self-associated state is more likely to have the capacity to accommodate a phenolic compound than the monomer. The hydropathy profile of insulinotropin (18) suggests that the highest hydrophobicity region lies between Ala-18 and Ala-24 (for the sequence of the peptide, see Material section). This region may play a role in forming a hydrophobic pocket to serve as the aromatic binding site of this peptide. However, identification of the definitive binding site for phenolic compounds requires more investigation.

Table III. Solubility of the Insulinotropin Suspensions in a pH 7.1 Phosphate Buffered Saline

Samples	Solubility (mg/ml)	Samples	Solubility (mg/ml)
I-A	0.45	IZ-B	0.02
IC-C	0.80	IZC-D	0.03

Macromolecular crystals typically have a high solvent content which ranges from 20–80% (28). The solvent commonly occupies large channels or interconnected interstitial voids which pass between the molecules. For that reason, crystalline macromolecules exhibit very few intermolecular bonds (salt, hydrogen, hydrophobic, etc.) in proportion to their gross size or weight (28). Since these crystal contacts provide the lattice interactions that maintain the integrity of the crystal, this in large part explains the weak lattice forces of macromolecular crystals. The high solvent content has the consequence of permitting diffusion of small molecules to any part of the surface of the macromolecule which is accessible in solution via the solvent channels (7–10). However, in some cases, lattice forces will not permit reactions to occur which would involve conformational changes or rearrangements of the macromolecule in the crystal. The disruption of the interactions which are responsible for intermolecular and crystal contacts may result in the cracking and dissolving of the crystals (8). In other cases, the forces that drive the conformational changes can be sufficient to overcome the constraints imposed by the crystalline lattice. These lattices may be more flexible and capable of accommodating limited conformational changes (7–10).

It is known that, in general, protein crystals grown from low ionic strength solutions are larger but less stable than those grown from high ionic strength solutions (8). The insulinotropin crystals grown in 1% NaCl (I-A) are believed to have a relatively weak lattice force. Upon the addition of m-cresol to suspension I-A to prepare suspension IC-C, m-cresol diffused into the I-A crystals through the solvent channels, and may have disturbed the crystal contacts. As a consequence, the crystals were rendered amorphous. On the other hand, upon the addition of zinc to suspension I-A to prepare suspension IZ-B, the crystals underwent a structural change but maintained their integrity. Zinc binding is believed to stabilize the crystal lattice of the original crystals. As a consequence, when m-cresol was added to suspension IZ-B to make suspension IZC-D, the particles remained crystalline, but underwent a structural change. The DSC data support this hypothesis. The quantity of heat absorbed in the transformation of solid to liquid is the heat of fusion (29). In condensed phases, intermolecular forces hold the molecules together. Usually, a slightly larger distance of separation between molecules exists in the liquid than the solid. The energy which is required to pull the molecules out of the ordered arrangement in the crystal to the disordered arrangement of the liquid is measured by the heat of fusion (29). Since the heat of fusion of sample IZC-D is considerably greater than that of IZ-B, it can be concluded that the lattice

force, the intermolecular force, or the crystal contact is greater for sample IZC-D than sample IZ-B.

As shown in Table III, the solubility of the solids from suspensions IZ-B and IZC-D was about the same in PBS. However, the solubility of the latter in its own suspension vehicle (containing NaCl, zinc, and m-cresol) was less than 1 μg/ml although that of the former in its own suspension vehicle (containing NaCl and zinc) was not much different from that in PBS (30). It is likely that when PBS was used for dissolution testing, m-cresol diffused into the dissolution medium from the IZC-D crystals through the solvent channels. This is supported by the washing experiment summarized in Table II. It seems that a significant concentration of free m-cresol in equilibrium with the bound cresol/insulinotropin is required in order to keep the cresol in the crystal lattice.

The pharmacokinetics (PK) of the four insulinotropin suspensions and a solution formulation of insulinotropin were studied in rats following subcutaneous administration (30). When compared to the solution, all the suspensions gave sustained release with distinct PK profiles. The ratio between the highest and lowest measurable plasma concentration (Cmax/Cmin ratio) in rats for the duration of the sampling interval (0.08–30 hours) was used as an index of sustained release. A lower Cmax/Cmin ratio implies more sustained release. It was found that suspension IZC-D had the lowest Cmax/Cmin ratio among the compared suspensions. The specific order of preparation appears to be important to achieve the most sustained release. When zinc and/or m-cresol were added to a saline solution of insulinotropin at the beginning of the crystallization, amorphous aggregates of insulinotropin formed instead of crystals. However, a suspension of insulinotropin crystals can be prepared in saline. Crystallinity can be retained by treating this suspension with zinc and then m-cresol.

ACKNOWLEDGMENTS

We wish to thank Mr. Douglas J. Allen (DSC and photomicrography), Mr. Stephen C. Maginess (^{19}F-NMR), and Ms. Debra L. De Costa (X-ray diffraction) for their technical assistance, and Dr. Jon Bordner for the discussion on the X-ray diffraction data. We would like to express our thanks also to Dr. Wayne A. Boettner who made helpful comments on the manuscript.

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