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Stabilization of insulin against agitation-induced aggregation by the GMO cubic phase gel

Yogesh Sadhale [1], Jaymin C. Shah *,[2]

Department of Pharmaceutical Sciences, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425, USA

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Abstract

The main objective of the study was to evaluate if the liquid crystalline cubic phase gel of glyceryl monooleate (GMO) protects insulin from agitation induced aggregation. The aggregation of Humulin®, Regular Iletin I® and Regular Iletin II®, in cubic phase GMO gels at 30 U/g of gel was compared with that in PBS at 100 oscillations/min at 37°C using optical density at 600 nm. The effect of agitation on the secondary structure of insulin in solution and in the gels was determined with circular dichroism (CD) spectroscopy, and the time course of aggregation was also followed by HPLC. A sigmoidal increase in optical density of solution with time indicated formation of increasing amounts of insoluble insulin aggregates. However, in the gels, optical density values stayed at, or around, the initial optical density value, comparable with that of a blank gel suggesting that insulin had not aggregated in the gel. CD spectroscopy of the soluble insulin showed a total loss of native conformation upon aggregation of insulin in solution. In contrast, CD spectra of insulin in the gel were unaltered suggesting protection from aggregation during agitation. Furthermore, agitation of insulin in gels for a duration as long as 2 months at 37°C, had very little adverse effect on the native conformation of insulin, as indicated by the lack of a significant change in its CD spectrum. Therefore, the cubic phase gel was indeed able to protect insulin from agitation-induced aggregation and subsequent precipitation. Although the majority of insulin in solution appeared to have aggregated and precipitated after 8 days by UV and CD spectroscopy, RP-HPLC results indicated the presence of some soluble aggregates of insulin. In summary, the liquid crystalline cubic phase gel of GMO protects peptides, like insulin, from agitation-induced aggregation.

Keywords: Cubic phase gel; GMO; Insulin; CD; Aggregation; Proteins

1. Introduction

Unlike conventional drugs, which degrade by chemical instability reactions such as hydrolysis, oxidation, deamidation, etc. peptide drugs are also inactivated by physical instability processes such as aggregation, precipitation, denaturation,

* Corresponding author. Tel.: +1-803-7925366; fax: +1-803-7920759.

E-mail address: shahjc@musc.edu (J.C. Shah)

[1] Present address: Pentech Pharmaceuticals Inc., 417 Harvester Court, Wheeling, IL 60090, USA.

[2] Present address: Pfizer Central Research, Pharmaceutical R & D, MS 8156-39, Eastern Point Road, Groton, CT 06340, USA.

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and surface adsorption (Arakawa and Timasheff, 1985; Wang and Hanson, 1988; Manning et al., 1989). Aggregation of insulin resulting in its precipitation has been a fundamental obstacle to the development of long-term delivery devices for insulin (Brange and Havelund, 1983; Brange and Langkjær, 1993). Various approaches have been used to protect protein and peptide drugs from physical inactivation, one of which has been the use of stability-enhancing additives (Wang and Hanson, 1988). In case of insulin, surfactants, dicarboxylic amino acids and phenol has been used to prevent its agitation-induced aggregation (Bringer et al., 1981; Wu and Yang, 1981; Chawla et al., 1985; Wollmer et al., 1987).

A large number of compounds like polysaccharides, polyols, amino acids, salts, etc. are known to stabilize proteins in solution by the excluded solute mechanism (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1988; Fágáin, 1995). Aggregation of proteins subsequent to conformational changes due to exposure to air–water and/or air–hydrophobic interfaces can be prevented using surfactants as stabilizers (Wang and Hanson, 1988). The objective of this study was to evaluate if insulin could be protected from agitation-induced aggregation using a glyceryl monooleate (GMO)–water cubic phase gel.

GMO, a polar lipid and a surfactant with a low hydrophilic–lipophilic balance (HLB) value, swells in water resulting in several gel phases, each composed of different liquid crystalline phases, including the cubic phase. The cubic phase consists of a curved continuous lipid bilayer extending in three dimensions, separating two congruent networks of water channels (Larsson, 1989; Ericsson et al., 1991). The water pore diameter of the fully swelled phase is ~ 5 nm and the cubic phase is extremely viscous (Larsson, 1989). This unique structuring of water and the high viscosity could lower the diffusion coefficient of water, which is an expression of its mobility in the gel. Ericsson et al. (1991) showed a reduction in diffusion coefficient of water and incorporated peptide in the cubic phase using NMR. Preliminary studies of diffusional release of tritiated water from cubic phase gel suggested a 30-fold reduction in diffusion coefficient of water in the cubic phase gel (Sadhale and Shah, 1996). Thus, due to the reduced translational mobility of protein and water, and the prevention of exposure of protein to air–water interface may protect an incorporated protein from agitation induced aggregation. The high viscosity may also prevent protein from aggregating by association and by reducing the effect of agitation. The above mentioned characteristics of the cubic phase could thus make it potentially useful as a physical stability enhancer of peptide drugs.

Although Ericsson et al. (1991) have demonstrated the ability of the cubic phase gel to protect an oligopeptide from enzymatic degradation, there have been no studies on its ability to protect peptides from physical instability. Therefore, the main objective of this study was to determine if cubic phase gel could protect peptides like insulin from physical instability such as aggregation. Insulin was chosen as a model peptide since it is known to aggregate upon agitation, the time course of which can be easily followed spectroscopically (Sluzky et al., 1991; Brange and Langkjær, 1993).

GMO gels have been suggested as delivery matrices for proteins and peptides, and the sustained delivery of oligopepetides such as desmopressin and somatostatin from GMO gel after subcutaneous administration has been demonstrated (Ericsson et al., 1991). Although cubic phase is highly viscous, GMO also forms lamellar phase gel of low viscosity with 3–20% water which can be injected through a syringe, and it is known to absorb water and body fluids in situ to convert into the cubic phase (Larsson, 1989). The biological activity of insulin in GMO gels will have to be demonstrated subsequent to these studies to demonstrate the use of GMO gels as vehicle not only for protein stabilization but also as delivery matrices.

2. Materials and methods

Humulin®, Regular Iletin I® and Regular Iletin II® were purchased from the MUSC Hospital Pharmacy. GMOrphic 80® (GMO, monoolein) was a generous gift from the Eastman Chemical

Company. Monobasic and dibasic sodium phosphates were obtained from Curtin Matheson Scientific, Inc. Methylene chloride and the disposable UV cuvettes were obtained from Fisher Scientific.

2.1. Preparation of the gels and solution

Gels containing 30 U of each insulin per gm of gel were prepared directly in disposable cuvettes. An accurately weighed (3.5 g), known quantity of GMO was warmed in a water-bath to 45°C until it melted. An appropriate amount (1.5 ml) of 100 U/ml insulin solution was then slowly added with stirring to the 3.5 g of molten GMO to form 30% water containing cubic phase gel which contained 30 U of insulin/g of gel. The gel formed immediately, which was then centrifuged to remove any air bubbles. The cubic phase was completely formed in less than 24 h and is characterized by a transparent, slightly golden color. The isotropic nature of the cubic phase and its phase transitions on heating were verified using hot-stage microscopy under polarized light (Larsson, 1989; Ericsson et al., 1991).

The three insulin solutions, Humulin®, Regular Iletin I® and Regular Iletin II®, each with a concentration of 100 U/ml (4 mg/ml), were diluted with 0.1 M phosphate buffer to obtain a final concentration of 30 U/ml (1.2 mg/ml), which were used in the agitation study as controls.

2.2. Agitation studies of insulin in cubic phase gel and solution

GMO cubic phase gels (GMO:insulin; 70:30 w/w) containing the three different insulins at a concentration of 30 U/g in disposable UV cuvettes were agitated at 100 oscillations/min in a water-bath shaker maintained at 37°C. Insulin solutions (30 U/ml) in cuvettes were subjected to the same agitation conditions and used as controls to compare rates of aggregation. Aggregation of insulin was monitored as a function of an increase in optical density of the gels and the solutions at 600 nm, as measured with a HP 8452A diode array spectrophotometer. The optical density values were plotted against time to obtain aggregation profiles.

2.3. Secondary structure of insulin by circular dichroism (CD) spectroscopy

To verify the integrity of the secondary structure of insulin in solutions and gels during the agitation study, another agitation study was conducted using the procedure describe above. The CD spectra of insulin (Humulin®) in the solution and gels that were agitated were obtained as described below. Six sets each, of insulin in solution and in gel, were prepared in the UV cuvettes as explained above. All the solutions and the gels were then agitated at 100 oscillations/min at 37°C. One set of solution and a gel was withdrawn daily and their optical density at 600 nm measured, to obtain aggregation profiles. Since the gel is viscous, it was softened by adding small amount of methylene chloride which dissolves the GMO leaving an aqueous solution of insulin which was then diluted with phosphate buffer to an insulin concentration of 0.2 mg/ml prior to analysis, assuming that all of insulin was in solution. This addition of small amounts of methylene chloride to GMO gel was confirmed previously to have no adverse effect on insulin in the gel by CD spectroscopy. Each test solution was then individually placed in a quartz cuvette having a path length of 0.1 cm and scans were conducted from a wavelength of 260 to 185 nm. Each profile represents the average of five scans of ellipticity θ (millidegrees) versus wavelength λ (nm). All scans were corrected for the baseline obtained by scanning a blank buffer solution. The CD spectra of insulin in solution and the diluted gels at various times were compared with the CD spectrum of intact insulin to determine structural integrity of insulin. The CD spectrum of insulin in a cubic phase gel that had been agitated for 2 months at 37°C was also obtained to further demonstrate the long duration protection of insulin against aggregation in the gel.

2.4. Aggregation and subsequent precipitation of Insulin in solution as studied by RP-HPLC

Effect of aggregation of insulin on its concentration in the solution was also monitored using an HPLC assay as described below. Humulin®

solution (100 U/ml) was diluted with 0.1 M, pH 7.4 phosphate buffer to obtain a solution containing 33.3 U/ml insulin. This solution was filled in three disposable UV cuvettes which were then agitated at 100 oscillations per minute at 37°C. At predetermined time intervals, the optical density at 600 nm of each solution was measured and then a 50 μl sample of the clear supernatant was withdrawn. When solutions in the cuvettes started to show signs of aggregation, they were centrifuged before withdrawing a sample from the clear supernatant. The samples were diluted to 4 ml with phosphate buffer and the amount of insulin remaining was determined by HPLC assay described below. The optical density values and the average insulin peak heights ($n = 3$) were plotted against time for evaluation of the aggregation process.

2.5. HPLC assay of insulin

Insulin was analyzed by reverse phase HPLC on a Hypersil™ C4 column (5 μm, 3.9 mm i.d × 300 mm) and eluted with the mobile phase at a flow rate of 1 ml/min. The mobile phase consisting of a mixture of 70% aq. solution of 0.02 M trichloroacetic acid and 30% acetonitrile, was filtered through a 0.45 μm membrane filter and deaerated under vacuum prior to use. Insulin peak was detected with an UV detector at 254 nm. Linear calibration plots were constructed for 0.625–10 μg/ml of insulin.

3. Results

3.1. Physical stability (agitation studies) of insulin in cubic phase gels and solutions

Fig. 1a–c show the aggregation profiles ($n = 3$) of Humulin®, Regular Iletin I® and Regular Iletin II® respectively, in pH 7.4 PBS and cubic phase gel, all of which were agitated at 100 oscillations/min at 37°C. The aggregation profile of Humulin solution shows a typical sigmoidal curve indicating a slow initial rise in optical density followed by a steep rise that gradually ends in a plateau. Similar profiles were obtained for solutions of Regular Iletin I and II. The increase in optical density at 600 nm is due to an increase in the turbidity of the solution which is caused by the formation of increasing amounts of insulin aggregates. There appears to be a lag phase (1–2 days) with a slow rise in optical density which is followed by an increase in the rate of aggregation, as seen by the rather sharp increase in optical density from day 2. Around day 6 or 7, when all the insulin appears to have aggregated, there is no further increase in the optical density of the solution indicating completion of the aggregation process. The slight decrease in optical density at the end of a week, seen in the aggregation profiles of Regular Iletin I and II, is due to the insoluble aggregates settling down in the solution. Effectively, optical density measures the loss of insulin from solution due to precipitation after aggregation.

In contrast to the aggregation profiles of insulins in solution, optical density of the gels (Fig. 1) throughout the study stayed at, or around, the initial low optical density value which was similar to that of a blank gel (gel without insulin). This indicates that insulin in the gel had not aggregated even upon agitation. Although the difference in optical density between the gels and solution may not appear significant at earlier times due to the higher initial optical density of gel (Fig. 1a–c), the increase in optical density was observed only for insulin solution. The cubic phase gel thus appeared to be able to protect insulin from agitation-induced aggregation at 37°C.

Although gel appeared to have protected insulin from aggregation, it was not known whether the native conformation, and particularly if the secondary structure of insulin was still intact in the gel. To address these questions, the secondary structure was assessed with CD spectroscopy.

3.2. Integrity of the secondary structure of insulin by CD spectroscopy

Fig. 2a and b show the CD spectra of insulin in solution and gels during the agitation study as compared to that of control insulin solution. In this experiment, aggregation profiles of insulin in

solutions and the gels were similar to the ones obtained in the previous agitation study (Fig. 1) indicating that insulin had indeed aggregated in solution but not in the gels.

The CD spectrum of control insulin solution prepared fresh (Fig. 2) in the far UV region (185–250 nm) showed extrema at 196, 209 and 222 nm with the ellipticity values crossing over to the negative side of the *X*-axis around 204 nm. This CD spectrum is in close agreement with the CD spectrum of insulin obtained previously by other investigators (Ettinger and Timasheff, 1971; Wu and Yang, 1981; Wollmer et al., 1987). The CD spectra of insulin in PBS solutions confirmed the earlier observations regarding aggregation of insulin (Fig. 2a). As compared to control insulin CD spectrum, no significant change in the CD spectrum of insulin in the agitated solutions was observed on day 2 which corresponded with a very minimal rise in optical density (1a–2a). On day 3, the spectrum showed no change in the spectral profile, but a decrease in the magnitude of the extrema at 196, 209 and 222 nm. This denotes the beginning of the aggregation process corresponding to loss of insulin from solution and an increase in UV optical density. There was a further decrease in the magnitude of the extrema in the spectrum for day 4 accompanied by a blue shift in the extremum at 196 nm and the *X*-axis crossover point to a lower wavelength. The spectra on days 6 and 7 showed a complete disappearance of the three extrema indicating a total loss of the native conformation which occurs prior to aggregation, and precipitation of insulin aggre-

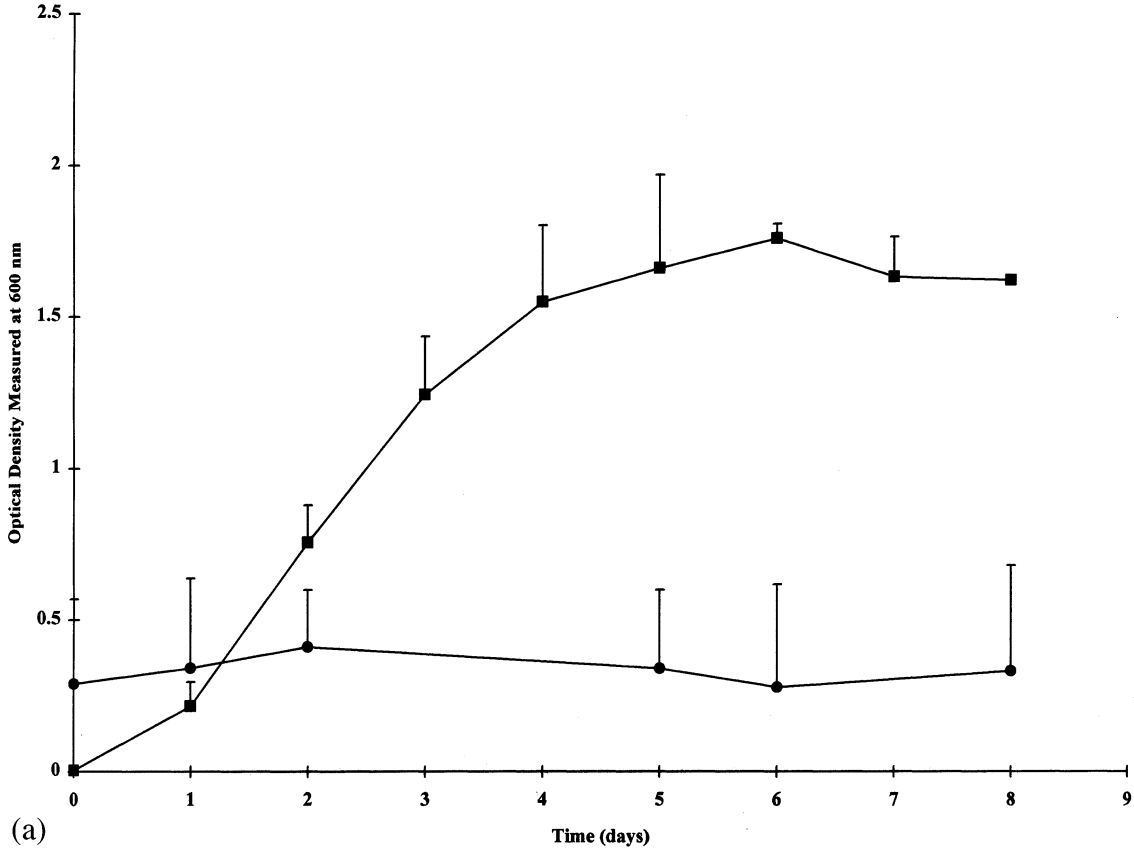


Fig. 1. Aggregation profiles of (a) Humulin®, (b) Regular Iletin I® and (c) Regular Iletin II® at pH 7.4 in PBS (■) and GMO gel (●) agitated at 100 oscillations/min at 37°C ($n = 3$).

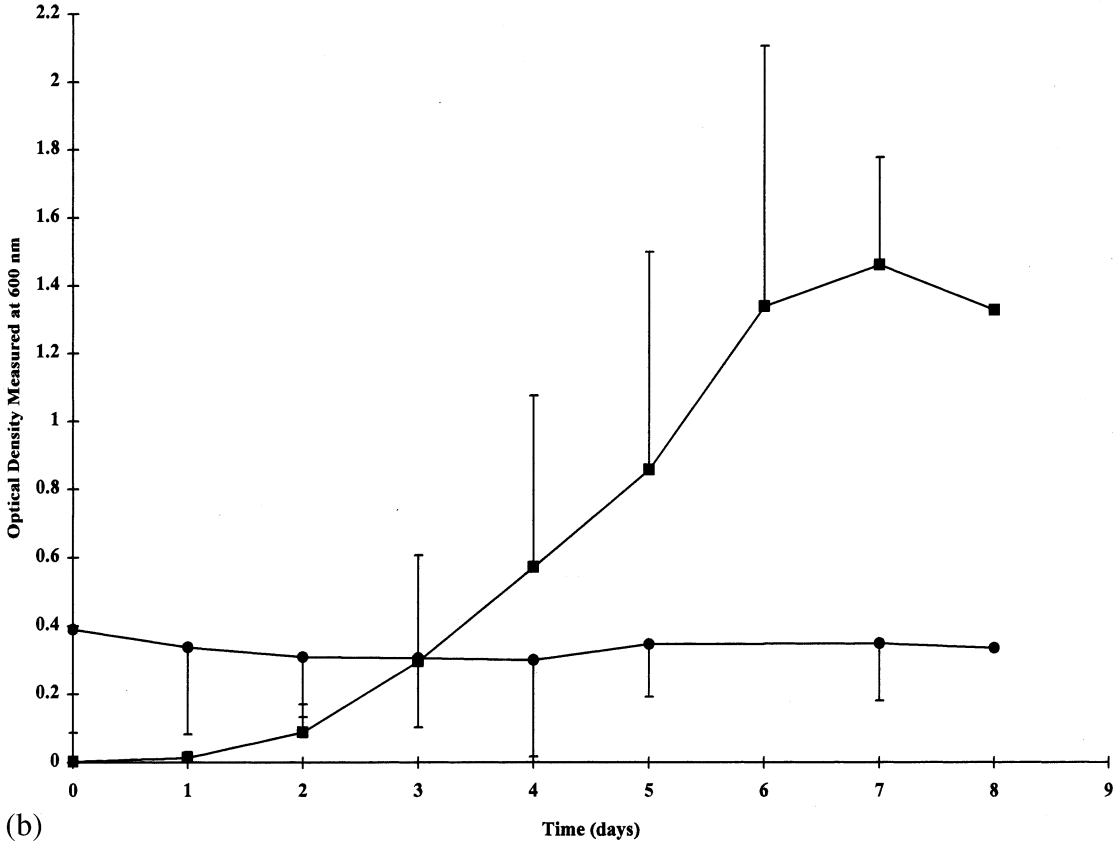


Fig. 1. (*Continued*)

gates from solution (Fig. 2a). Since the CD spectra were obtained from diluted clear supernatant, only changes in soluble insulin due to unfolding and soluble aggregate formation could be observed.

The CD spectra of insulin in the gels (Fig. 2b) show that insulin was unaffected by agitation. All the spectra displayed the expected three extrema at 196, 209 and 222 nm. All the spectra were essentially overlapping and very similar to that of control insulin solution except for slight differences in the magnitudes of their extrema (Fig. 2b). When the ellipticity values at the peak extrema of insulin solution and gels were plotted as a function of time of agitation (Fig. 3a and b), in solution, a sigmoidal change in ellipticity was observed at all three peak extrema, 196, 209 and 222 nm, eventually the ellipticity becoming negligible, suggesting total loss of secondary structure in solution. In contrast, ellipticity for insulin in gel at all three-peak extrema remained relatively constant without a trend suggesting no change (Fig. 3a and b).

The CD spectrum of insulin (Fig. 4) in a gel that had been agitated for 2 months also shows very little change in the secondary structure when compared to the CD spectrum of control insulin (Fig. 2). Thus, the absence of any significant change in the CD spectra over a period as long as 2 months seems to indicate that neither the agitation of insulin in the gel nor the procedure for diluting the gel had any adverse effect on the native conformation of insulin. Therefore, insulin was indeed protected from agitation induced aggregation by GMO cubic phase gel for a period as long as 2 months.

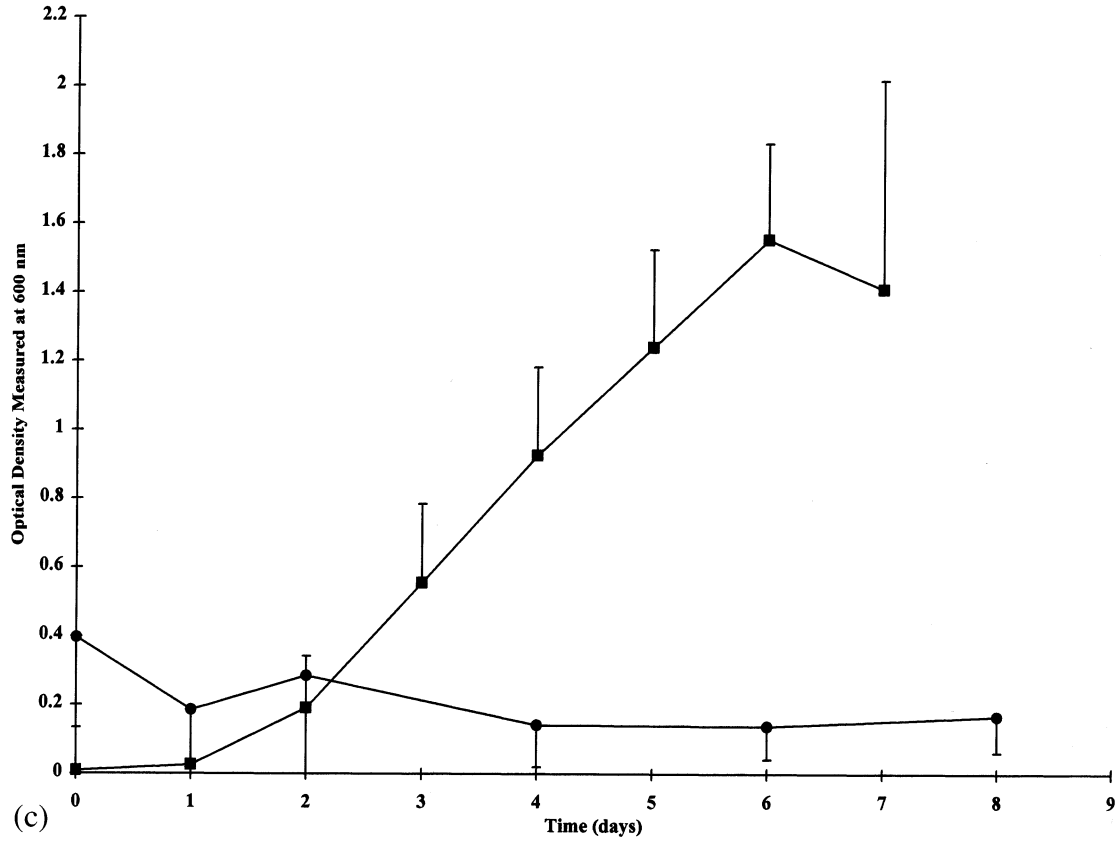


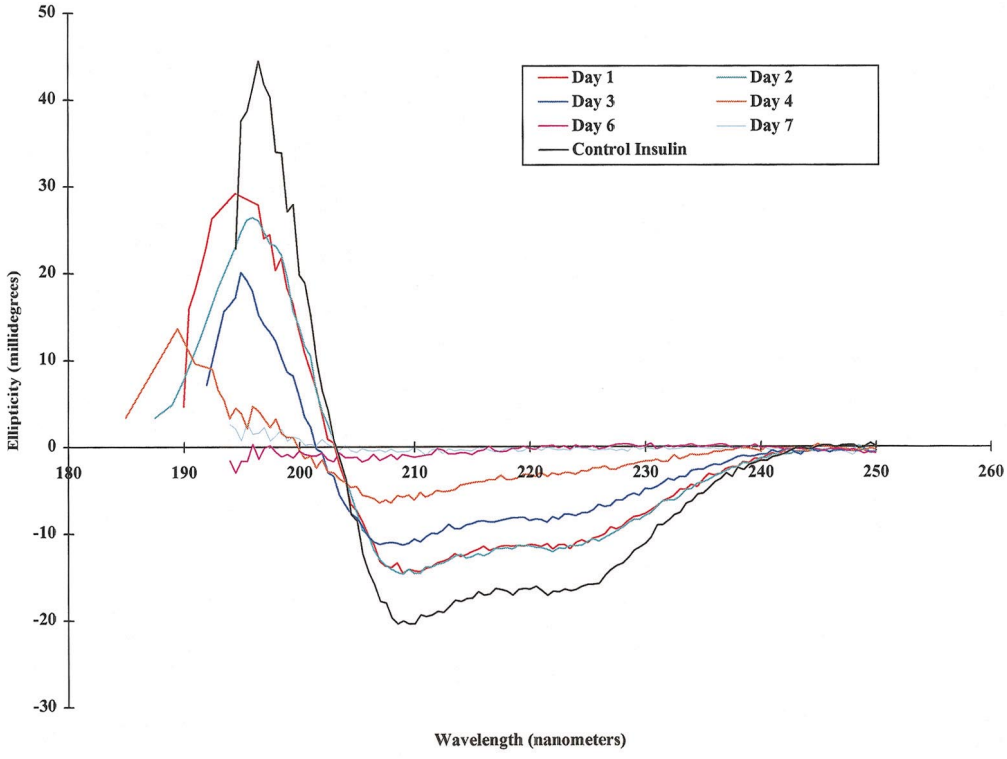
Fig. 1. (*Continued*)

Since insulin in the agitated solution appeared to have completely aggregated, it was logical to follow the process chromatographically whereby insulin could be separated from its insoluble aggregates and the time course of the aggregation followed to the point where the chromatographic peak for insulin could no longer be observed. It would be useful to know if the total loss of secondary structure coincides with aggregation and the resulting precipitation of all of insulin from solution. Therefore, another study was conducted in which aggregation of insulin in solution was simultaneously followed by CD, UV/Vis spectroscopy and reverse phase HPLC.

3.3. Aggregation of insulin as studied by HPLC

Fig. 5 shows the aggregation profile of insulin and a plot of average peak height (cm) of the chromatographic peak of insulin versus time. The aggregation profile followed a typical sigmoidal curve as was observed in the earlier study. As a result of agitation the insulin precipitated, giving lower peak heights for soluble insulin by HPLC. Since by optical density measurements and CD, all the insulin in solution appeared to have precipitated after 8 days, the concentration and consequently, the average peak height of insulin should have decreased to 0. However, as seen in Fig. 5, the insulin peak could still be observed, and therefore, there may be some soluble denatured insulin or soluble aggregates that could not be resolved from the peak for the native unaggregated insulin by RP-HPLC. Also, the mobile phase of the RP-HPLC could have dissociated the soluble aggregates and/or renatured the insulin resulting in a peak corresponding to native insulin.

(a)


Day 1
Day 2
Day 3
Day 4
Day 6
Day 7
Control Insulin
Ellipticity (millidegrees)
50
40
30
20
10
0
-10
-20
-30
180
190
200
210
220
230
240
250
260
Wavelength (nanometers)

(b)

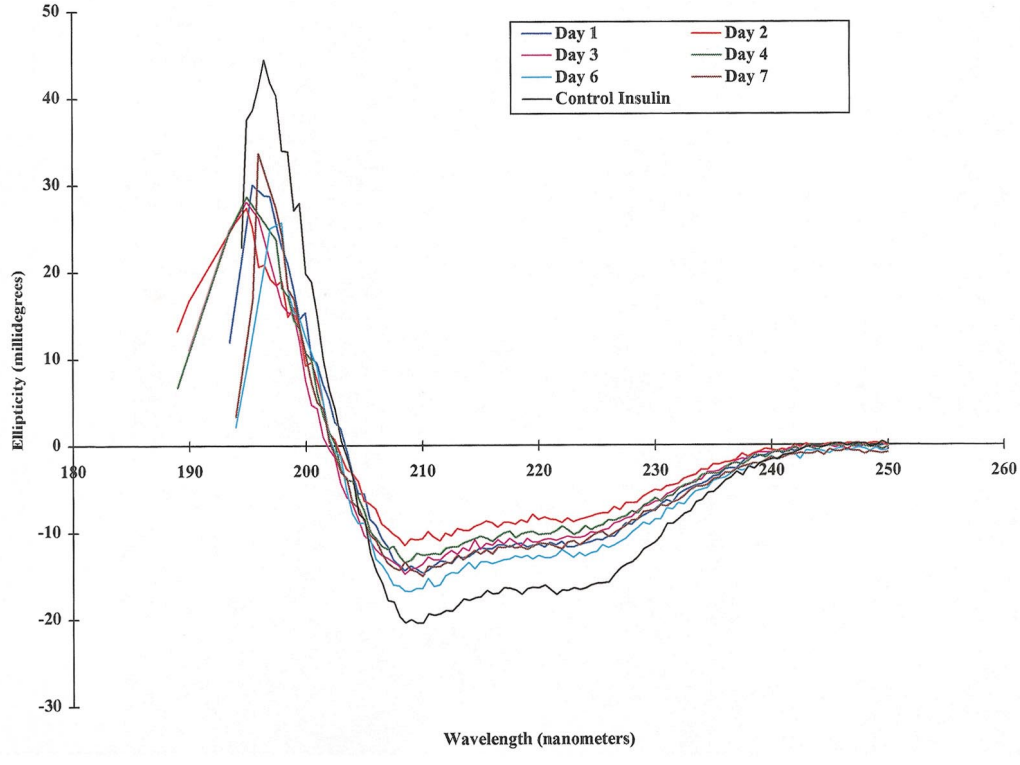

Day 1
Day 2
Day 3
Day 4
Day 6
Day 7
Control Insulin
Ellipticity (millidegrees)
50
40
30
20
10
0
-10
-20
-30
180
190
200
210
220
230
240
250
260
Wavelength (nanometers)

Fig. 2.

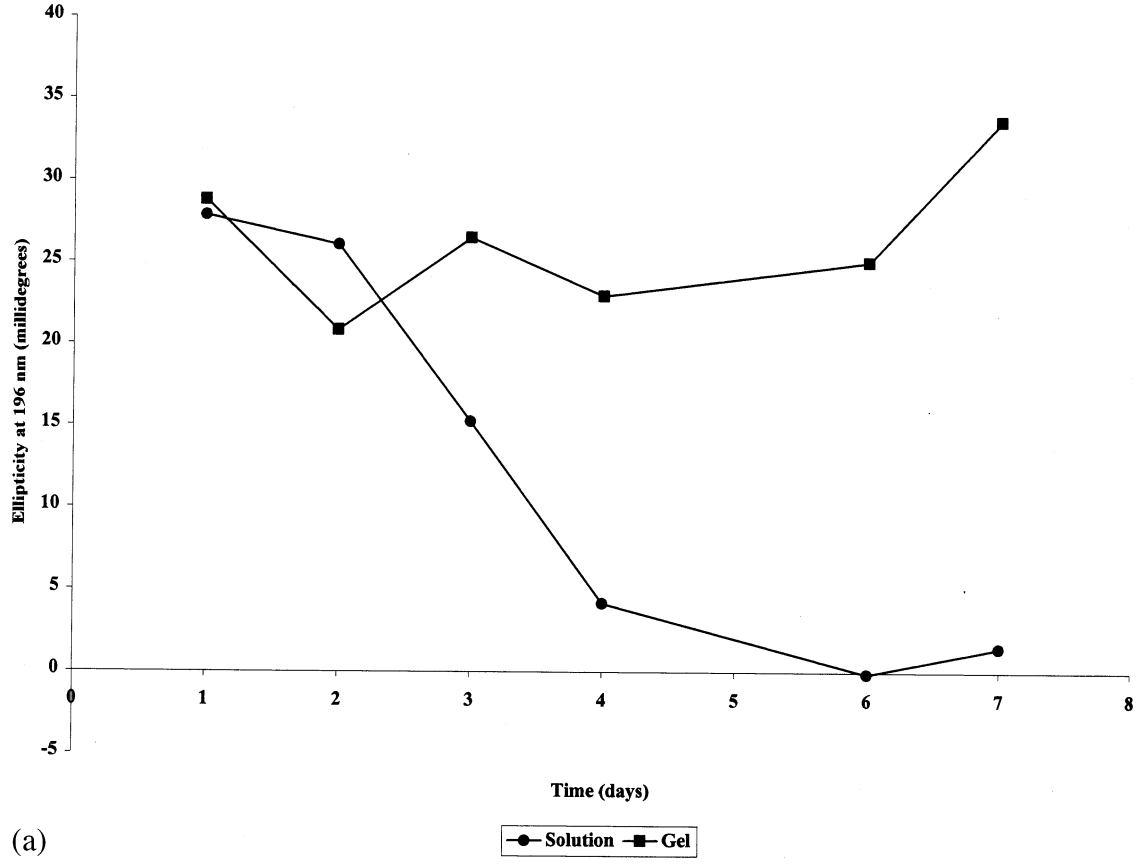


Fig. 3. Changes in the ellipticity values at the extrema at (a) 196 nm and (b) 209 and 222 nm in the circular dichroic spectra of insulin in solution and gel when both were agitated at 100 oscillations/min at 37°C for 7 days.

4. Discussion

Insulin was used as the model protein because it has been extensively studied for its proclivity to aggregate and precipitate leading to a drastic reduction in its biological potency (Bringer et al., 1981; James et al., 1981; Brange and Havelund, 1983; Lougheed et al., 1983; Brennan et al., 1985; Chawla et al., 1985). Agitation and high temperatures are known to accelerate the precipitation of insulin out of solution. Aggregation and the subsequent precipitation of insulin upon agitation has been shown to occur through the intermediate step of fibril formation (Waugh et al., 1953; Brange and Langkjær, 1993). The fibrils grow further to form larger aggregates and this finally leads to precipitation as described in detail by Brange and Langkjær (1993). The initial slow rise in optical density, or the so called lag phase (1–2 days) observed in this study, is due to the time it takes to obtain stable fibril nuclei which are able

Fig. 2. Changes in the circular dichroic spectra of insulin in (a) solution and (b) gel, as compared with control insulin, when both were agitated at 100 oscillations/min at 37°C for 7 days. The CD spectrum of control insulin (Humulin®) consists of three extrema at 196, 209 and 222 nm, and each spectrum is an average of five scans of sample with a pathlength of 0.1 cm. The samples were prepared by dilution to contain 0.2 mg/ml protein assuming all insulin was in solution, however, the actual insulin concentrations may be lower due to precipitation of insulin aggregates from solution.

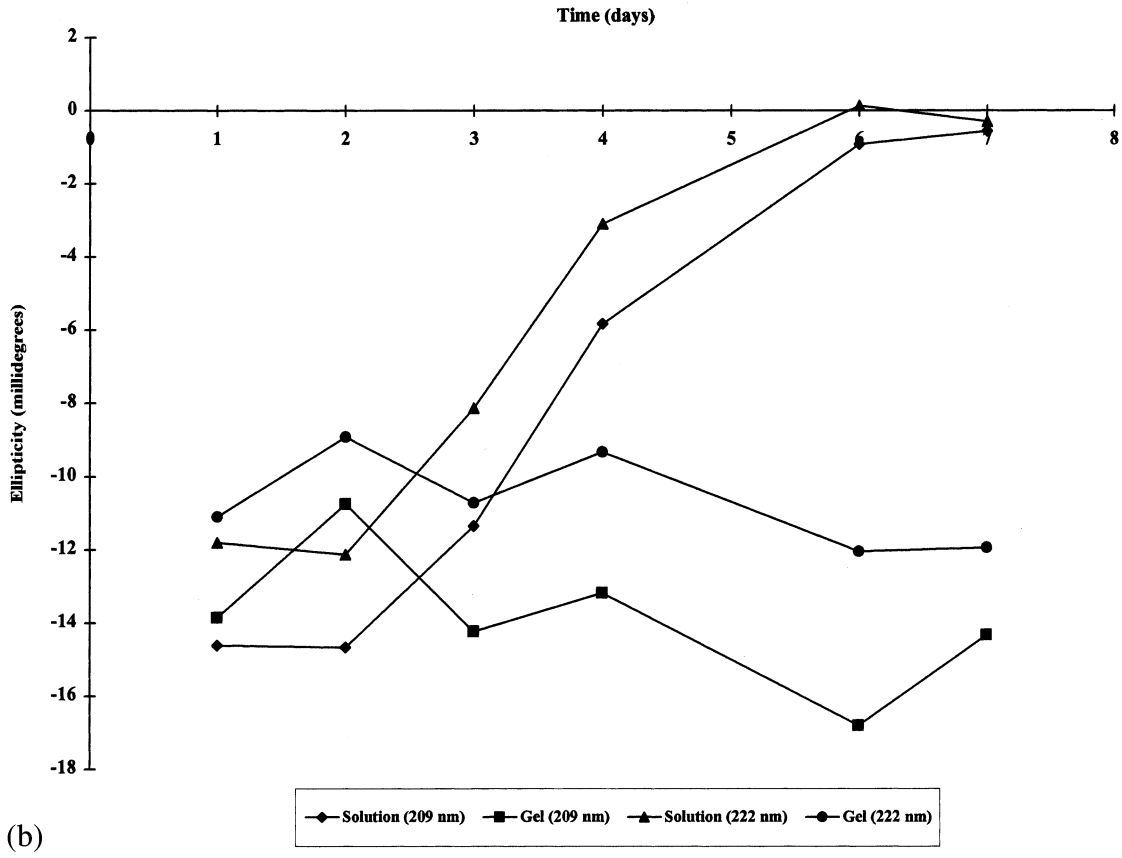


Fig. 3. (*Continued*)

to start subsequent aggregation (Dathe et al., 1990; Sluzky et al., 1991; Brange and Langkjær, 1993). When sufficient amounts of fibril nuclei are accumulated there is an increase in the rate of aggregation, as seen by the rather sharp rise in optical density from day 2. When all the insulin aggregated and precipitated there was no further increase in the turbidity of the solution resulting in a plateau in the aggregation profile. However, optical density of insulin in gel remained steady around the initial value, suggesting that insulin had not aggregated in gel.

Although the gel appeared to have protected insulin from agitation-induced aggregation, it was not known whether the native conformation of insulin was still intact, therefore the secondary structure was probed using CD spectroscopy. The CD spectrum of control insulin solution (Fig. 2) in the far UV region (185–250 nm) shows extrema at 196, 209 and 222 nm with ellipticity values crossing over to the negative side of the X-axis at around 204 nm. The extremum at 196 nm results from a contribution by the β-conformation in addition to the optical activity associated with other conformations while the extrema at 209 and 222 nm result from contributions from the appreciable amount ($\approx$ 20–40%) of α-helical structure in insulin (Ettinger and Timasheff, 1971). The CD spectra of insulin in agitated solutions confirmed the earlier observations regarding the aggregation behavior of insulin. The CD spectra of the agitated insulin solutions (Fig. 2a) on day 3 show a slight decrease in the magnitude of the three extrema indicating the beginning of the aggregation process, as also seen by a rise in optical density at 600 nm. The further decrease in

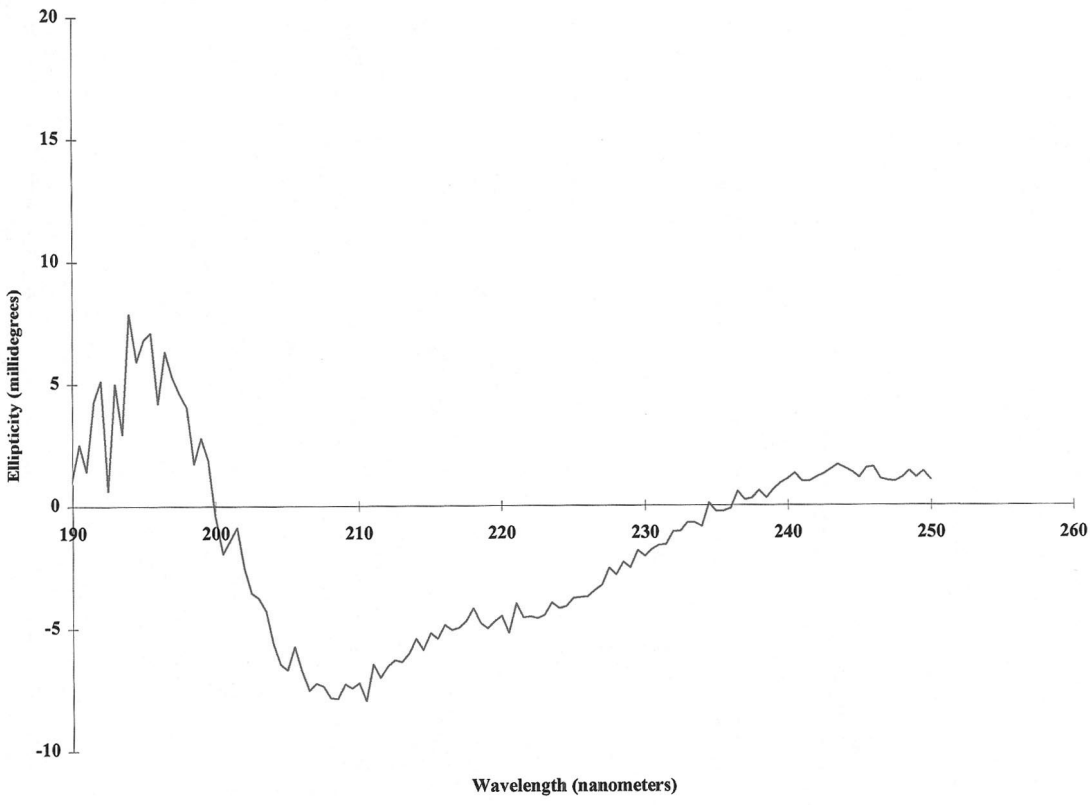


Fig. 4. The CD spectrum of insulin from a GMO cubic phase gel that was agitated for 2 months at 100 oscillations/min at 37°C. The spectrum is an average of five scans of sample with a pathlength of 0.1 cm.

the magnitude of the extrema in the spectrum for day 4 and the blue shift of the extremum at 196 nm and the *X*-axis crossover point is caused by the beginning of the chain unfolding process and the loss of unfolded and aggregated insulin from solution. This causes a further decrease in ellipticity which is reflected in a decrease in the magnitude of the three extrema. The complete disappearance of the three extrema in the spectra on days 6 and 7 is indicative of total loss of native conformation that could have led to aggregation and loss of insulin from solution (Fig. 3a and b). Also, the sigmoidal change in ellipticity and reaching a value close to zero parallels the sigmoidal increase in optical density followed by a plateau observed in earlier experiments suggesting these observations were due to same phenomenon of aggregation of insulin and loss of insulin from solution (Figs. 1 and 3).

The lack of any significant change in the CD spectra of insulin from the gels shows that this insulin was unaffected by agitation (Fig. 2b). All the spectra were overlapping and display the expected three extrema at 196, 209 and 222 nm compared with the control. Similar CD spectrum was obtained of insulin in a gel that had been agitated for a duration as long as 2 months (Fig. 4). Thus, the absence of any significant change in the CD spectra over a period as long as 2 months indicates that GMO cubic phase gel was able to protect insulin against agitation induced aggregation and maintain it in its native conformation. The results of this study also showed that neither the gel nor the use of methylene chloride to soften

the gel adversely affected the secondary structure of insulin.

Surfactants have been shown to prevent conformational changes in a protein by preventing their exposure to water–air and/or water–hydrophobic interfaces (Arakawa and Timasheff, 1985; Wang and Hanson, 1988; Manning et al., 1989). Since GMO is a surfactant with the ability to form lipid bilayers, it can similarly protect an incorporated protein from undergoing conformational changes due to exposure to water–air interfaces. Furthermore, the high viscosity of the gel by reducing the translational mobility of insulin may result in reduced collision frequencies and thus prevent aggregation by association. Effectively, insulin in the gel does not experience the stress of agitation which in solution leads to aggregation due to increased exposure to the air–water interface catalyzing a conformational change. It has been shown that shear stresses are not responsible for aggregation of growth hormone and proteins when exposure to air–water interface is prevented as in the case of insulin in the gel (Sluzky et al., 1991; Maa and Hsu, 1998). Osmolytic additives like sugars, polyols, amino acids etc., when added to proteins in solution, are preferentially excluded from the protein domain leading to the selective hydration of the protein (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1988; Fágáin, 1995). Water forms a hydration layer around the protein stabilized by a combination of ionic, hydrophobic and hydrophilic interactions

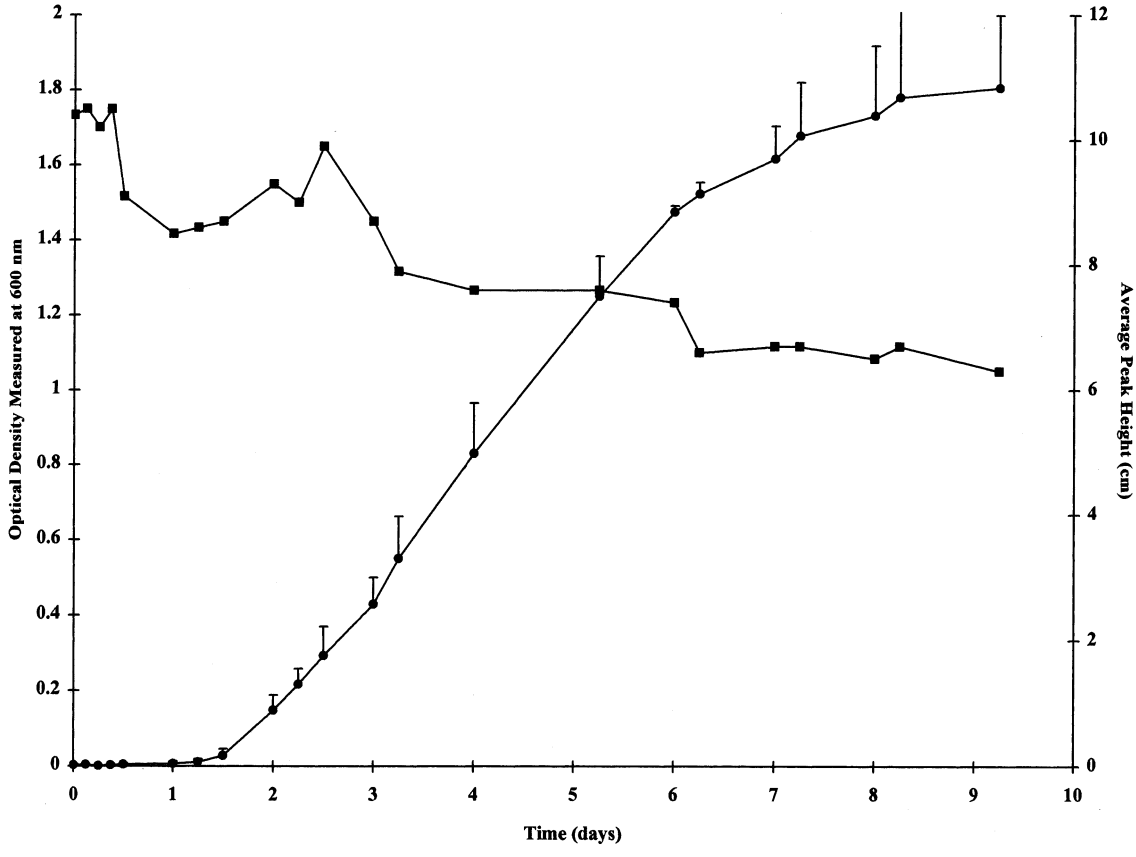


Fig. 5. Aggregation of insulin (33.33 U/ml) on agitation (100 oscillations/min at 37°C) resulting in its precipitation as seen by a decrease in its concentration (■) (determined by HPLC) along with an increase in optical density at 600 nm (●) suggesting insoluble aggregate formation ($n = 3$).

(Frauenfelder and Gratton, 1986). Although the chemical potential of both the native and unfolded protein are destabilized by the negative binding of the ligand, unfolded protein is destabilized to a greater extent by the excluded solute due to the increased exposed surface area of the protein. Therefore, the free energy required to unfold the protein is increased thus resulting in net stabilization by the excluded solute. The solute exclusion and the hydration layer around the protein resists such a thermodynamically unfavorable conformational change resulting in the stabilization of the protein by the Arakawa–Timasheff mechanism (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1988). Similarly, in the cubic phase gel, water with reduced mobility could form a hydration layer around insulin, thus preventing it from unfolding. The structuring of water in the cubic phase could result in a reduction of its mobility relative to that of bulk water as suggested by Sansom et al. (1996), who by mathematical modeling of water dynamics in channel like cavities showed that for cavities of 3–6 Å radii, water mobility was reduced relative to that of bulk water. Thus, a combination of different mechanisms may be responsible for stabilization of insulin in the gel.

Although all of insulin in solution appeared to have lost secondary structure, aggregated and precipitated, as one would conclude from the plateau in optical density seen at the end of the study, the average peak height of insulin did not fall down to 0 (Fig. 5). Since aggregation follows unfolding of insulin, and this loss of the native conformation is reflected in a change in the CD spectrum, it is unlikely that there is any native insulin left in solution. However, some of the unfolded or non-native insulin could still exist as soluble aggregates which may not be resolved from intact insulin by reverse phase HPLC, and thus the peak may be due to the presence of soluble aggregates. Also, the mobile phase of the RP-HPLC could have dissociated and/or renatured the soluble aggregates resulting in a peak corresponding to native insulin. The presence of soluble aggregates could have been identified using dynamic light scattering on the supernatant before RP-HPLC. Based on the composite results of all these studies one concludes that neither spectroscopic techniques, such as UV or CD, nor chromatography alone can be used to follow an aggregation process for proteins. However, a combination of CD, UV spectroscopy and chromatography provides a more comprehensive understanding of the aggregation process.

In conclusion, GMO cubic phase gel was indeed successful in stabilizing insulin against agitation-induced aggregation. Although the gel protected insulin from aggregation, it is unknown whether it is still biologically active. Therefore the biological activity of insulin in the gel needs to be evaluated to determine the suitability of the cubic phase gel as a stability enhancer and a delivery matrix for insulin.

Acknowledgements

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References

Arakawa, T., Timasheff, S.N., 1985. Mechanism of poly(ethylene glycol) interaction with proteins. Biochemistry 24, 6756–6762.

Brange, J., Havelund, S., 1983. Insulin pumps and insulin quality: requirements and problems. Acta Med. Scand. Suppl. 671, 135–138.

Brange, J., Langkjær, L., 1993. Insulin structure and stability. In: Wang, Y.J., Pearlman, R. (Eds.), Stability and Characterization of Protein and Peptide Drugs: Case Histories. Plenum Press, New York, pp. 315–350.

Brennan, J.R., Gebhart, S.S., Blackard, W.G., 1985. Pump-induced insulin aggregation. A problem with the Biostator. Diabetes 34, 353–359.

Bringer, J., Heldt, A., Grodsky, G.M., 1981. Prevention of insulin aggregation by dicarboxylic amino acids during prolonged infusion. Diabetes 30, 83–85.

Carpenter, J.F., Crowe, J.H., 1988. The mechanism of cryoprotection of proteins by solutes. Cryobiology 25, 244–255.

Chawla, A.S., Hinberg, I., Blais, P., Johnson, D., 1985. Aggregation of insulin in solution containing surfactants, in contact with different materials. Diabetes 34, 420–424.

Dathe, M., Gast, K., Zirwer, D., Welfle, H., Mehlis, B., 1990. Insulin aggregation in solution. Int. J. Peptide Protein Res. 36, 344–349.

Ericsson, B., Eriksson, P.O., Lofroth, J.E., Engstrom, S., 1991. Cubic phases as delivery systems for peptide drugs. ACS Symposium Series No. 469. In: Dunn, R.L., Ottenbrite, R.M. (Eds.), Polymeric Drug and Drug Delivery Systems. American Chemical Society, Washington, DC, pp. 251–265.

Ettinger, M.J., Timasheff, S.N., 1971. Optical activity of insulin I. On the nature of the circular dichroism bands. Biochemistry 10, 824–840.

Fágáin, C.O., 1995. Understanding and increasing protein stability. Biochim. Biophys. Acta 1252, 1–14.

Frauenfelder, H., Gratton, E., 1986. Protein dynamics and hydration. Methods Enzymol. 127, 207–216.

James, D.E., Jenkins, A.B., Kraegen, E.W., Chrisholm, D.J., 1981. Insulin precipitation in artificial infusion devices. Diabetologia 21, 554–557.

Larsson, K., 1989. Cubic lipid–water phases: structure and biomembrane aspects. J. Phys. Chem. 93, 7304–7314.

Lougheed, W.D., Albisser, A.M., Martindale, H.M., Chow, J.C., Clement, J.R., 1983. Physical stability of insulin formulations. Diabetes 32, 424–432.

Maa, Y.F., Hsu, C.C., 1998. Investigations on fouling mechanisms for recombinant human growth hormone during sterile filtration. J. Pharm. Sci. 87, 808–812.

Manning, M.C., Patel, K., Borchardt, R.T., 1989. Stability of protein pharmaceuticals. Pharm. Res. 6, 903–918.

Sadhale, Y., Shah, J., 1996. The effect of water-mobility and viscosity on the physical stability of insulin in a glyceryl monooleate–water cubic phase gel. Pharm. Res. 13 (9), 92S.

Sansom, M.S., Kerr, I.D., Breed, J., Sankararamakrishnan, R., 1996. Water in channel-like cavities: structure and dynamics. Biophys. J. 70, 693–702.

Sluzky, V., Tamada, J.A., Klibanov, A.M., Langer, R., 1991. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. Proc. Natl. Acad. Sci. USA 88, 9377–9381.

Wang, Y.-C.J., Hanson, M., 1988. Parenteral formulations of proteins and peptides: stability and stabilizers. J. Parenteral Sci. Tech. 42, S3–S25.

Waugh, D.F., Wilhelmson, D.F., Commerford, S.L., Sackler, M.L., 1953. Studies on the nucleation and growth reactions of selected types of insulin fibrils. J. Am. Chem. Soc. 75, 2592–2600.

Wollmer, A., Rannefeld, B., Johansen, B.R., Hejnaes, K.R., Balschmidt, P., Hansen, F.B., 1987. Phenol-promoted structural transformation of insulin in solution. Biol. Chem. Hoppe-Seyler 368, 903–911.

Wu, C.-S.C., Yang, J.T., 1981. Conformation of insulin and its fragments in surfactant solutions. Biochim. Biophys. Acta 667, 285–293.