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Probing the mechanism of insulin aggregation during agitation

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ABSTRACT

Agitation of insulin during its storage and transport has been reported to denature the protein, resulting in its inactivation. The physical changes accompanying the denaturation process which result in aggregation of insulin are poorly understood. In this work, we study the aggregates formed following agitation of insulin under defined conditions by different physical and chemical techniques. We show that both non-disulphide-mediated covalent bonds as well as non-covalent interactions are involved in the formation of aggregates. The two-step kinetics of aggregation could be clearly seen, with discrete 'early' and 'late' stages of fibrillation. Understanding the nature of agitation-induced aggregates will help in devising a strategy to protect this therapeutic protein against instability during its storage and transport.

1. Introduction

Misfolding of proteins, resulting in the formation of β-sheet-rich amyloid fibrils, is a characteristic feature of many neurodegenerative diseases and also in other cases where protein instability is a concern, as in the production and storage of therapeutic proteins (Wang, 2005; Wang et al., 2010). Proteins like lysozyme (Hevehan and Clark, 1997), truncated form of tumour necrosis factor-α (TNF-α) (Peternel et al., 2008) and some of the recombinant human growth hormones (Patra et al., 2000) have been reported to aggregate during their production stages, forming cross β-sheet structures. Like many therapeutic proteins which are used in solution, insulin poses a challenge to stabilization scientists. Although the production of insulin is no longer a limiting parameter, the bioavailability of the molecule still depends on a lot of factors. These include the stability of the protein during production, packaging, storage and transport. Thus, it is important to understand the nature of changes that insulin undergoes during these processes and the underlying mechanism involved therein. Aggregation of insulin in vials, infusion pumps and controlled release devices leads to reduction in its biological availability (Brennan et al., 1985; Renard, 2002). One of the main causes of aggregation in these devices is via agitation. Agitation leads to a realignment of the protein molecule at the air–water interface, leading to exposure of the inner hydrophobic core of the protein to air, which acts as the nucleus to induce aggregation (Sluzky et al., 1991). The exposure of the hydrophobic core leads to the aggregation of unfolded protein chains which eventually increases the size and number of the aggregated species. The extent of aggregation has been found to increase with the hydrophobicity of the surface and surface area (Sluzky et al., 1991). Understanding the nature of aggregates could provide a solution to the use of additives in stabilizing such dosage formulations (Philo and Arakawa, 2009).

A majority of previous reports have addressed the problem of chemical instability of insulin. Approaches to tackle these degradation routes have been proposed, and in many cases, promising solutions have been put forth. Studies have also concentrated on fibrillar nature of insulin aggregates (Hammarström et al., 2010; Heldt et al., 2011; Yang et al., 2010). The problem of physical instability however has largely remained ignored. Chemical modifications such as disulphide scrambling and deamidation have been shown to lead to physical changes such as aggregation and denaturation (Wang, 2005). Physical degradation of proteins thus continues to remain an area of concern. Some reports are available regarding instability of insulin at the air–water interface, at hydrophobic surfaces *per se*, high temperatures and in pump materials (Brennan et al., 1985; Renard, 2002; Arora et al., 2004). However, the nature of the aggregates formed has not been rigorously described. In this work, we report the detailed nature of the aggregates formed when insulin solution, composed of mainly monomeric/dimeric insulin and free of any preformed aggregates, is subjected to agitation.

2. Materials and methods

Insulin (from bovine pancreas, ≥95% HPLC), was purchased from Sigma–Aldrich, Bangalore, India. All other reagents used were of analytical grade or higher.

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2.1. Preparation of samples

Zn-free insulin was prepared by using a previously described method (Costantino et al., 1994) and has been used throughout the study. Insulin solution was freshly prepared in sodium phosphate buffer (0.05 M, pH 7.4) at a concentration of 0.5 mg ml^{-1}. The concentration of insulin was determined using an extinction coefficient (1 mg ml^{-1}) of 1 at 276 nm (Nielsen et al., 2001a). 1 ml insulin solution was aliquoted into 10 ml glass vials each, and placed vertically in an incubator shaker at 37 °C with shaking at 300 rpm. The sample vials were withdrawn and analyzed in triplicates at different time points of study. Control samples at different time points refer to samples incubated without agitation. The amount of protein initially subjected to agitation has been considered to be 100%.

2.2. Measurement of optical density of agitated samples

Aliquots were withdrawn from the vial at different time intervals and the absorbance of the samples was determined at 600 nm and taken to be a measure of turbidity of the agitated suspensions. Control samples at different time points refer to samples incubated without agitation.

2.3. Chromatographic measurements

Aliquots were withdrawn from the vials at different time intervals and centrifuged at 13,000 × *g* for 5 min. The supernatant was analyzed spectrophotometrically at 276 nm. The supernatant was filtered through 0.2 μm polyethersulphone membrane filter and loaded on a C18 column (Zorbax 300SB-C18) or SEC-HPLC column (Zorbax GF-250) attached to an HPLC system operated at room temperature. The mobile phase for RP-HPLC consisted of 30% (v v^{-1}) acetonitrile and 0.1% (v v^{-1}) trifluoroacetic acid at a flow rate of 0.5 ml min^{-1}. The column eluates were monitored online at 214 nm using a photodiode array detector (SPD-M20A). All absorbance signals were quantified by integrating the peak of interest using the software LC solution version 1.22 SP1 supplied by the manufacturer. The sample incubated for 180 min without agitation was used to confirm that no change in particle size occurred during the incubation period.

2.4. Dynamic light scattering (DLS) and zeta potential studies

Aliquots were withdrawn from the vials at different time intervals. The average particle size was measured by dynamic light scattering by the samples using Zeta sizer (Nano ZS, Malvern Instruments) at 25 °C. The measurements were done in zeta sizing cuvette by collecting 5 scans for each sample in triplicates. The autocorrelation function of the scattered light was fitted by cumulant analysis which showed the decay time of the particles. The sample incubated for 60 min without agitation was used as the control to confirm that no change in particle size occurred during the incubation period. Zeta potential of the samples was also measured by using zeta cell using the same instrument in a similar manner.

2.5. Estimation of free amine groups

The number of free amine groups in the insulin samples was estimated using 2,4,6-trinitrobenzene sulphonic acid (TNBSA) assay (Snyder and Sobocinski, 1975). Quantitative determination of the number of free amine groups in the samples was carried out using L-alanine as the standard molecule. Control samples at different time points refer to samples incubated without agitation.

2.6. Estimation of free sulphydryl groups

Estimation of free sulphydryl groups was carried out using Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB)] (Riddles et al., 1983). DTNB (Ellman's reagent) reacts with a free sulphydryl group to yield mixed disulphide and 2-nitro-5-thiobenzoic acid (TNB). The amount of sulphydryl groups was calculated by measuring the intense absorbance of TNB dianion at 412 nm and using the molar extinction coefficient of TNB equal to 14,150 M^{-1} cm^{-1} (Eyer et al., 2003). Control samples at different time points refer to samples incubated without agitation.

2.7. Native polyacrylamide gel electrophoresis

The agitated insulin sample was centrifuged (13,000 × *g* for 5 min) after 180 min. The pelleted aggregates were suspended separately in sodium phosphate buffer (0.05 M, pH 7.4), 8 M urea and 10 mM DL-dithiothreitol (DTT). The samples were incubated for 20 min at room temperature and the composition of the samples was checked by native polyacrylamide gel electrophoresis (Laemmli, 1970). 15% crosslinked polyacrylamide gel was used and 20 μg of protein was loaded in each well. The gel was silver stained to visualize the protein (Switzer et al., 1979).

2.8. MALDI-TOF mass spectrometry

The agitated sample obtained after 60 min was centrifuged at 13,000 × *g* for 5 min to separate the soluble and insoluble fractions. The pellet obtained was suspended in deionised water and 6 M urea separately. The aggregates suspended in 6 M urea were incubated at room temperature for 1 h, centrifuged at 13,000 × *g* for 5 min and then dialyzed against deionised water for 3 h. The mass spectra of the protein samples thus obtained in the supernatants (soluble fraction after aggregation and after solubilisation in 6 M urea), along with that of the control protein sample, were acquired on MALDI TOF/TOF mass spectrometer (Ultraflex, Bruker Daltonics). For acquisition of a mass spectrometric peptide map, 1:1 ratio of sample and matrix (saturated solution of α-cyano 4-hydroxy cinnamic acid in 33% (v v^{-1}) acetonitrile and 0.1% (v v^{-1}) trifluoracetic acid) was premixed and spotted onto a MALDI target plate. The acquisition operation mode was reflector mode and accelerating voltage was 25,000 V.

2.9. Thioflavin T (ThT) assay

ThT is a cationic benzothiazole dye that binds to amyloid fibrils with crossed β-sheet structures and exhibits a drastic enhancement of its emission maxima. Aliquots (50 μl) were withdrawn from vials at different time intervals and added to 25 μM ThT in sodium phosphate buffer (0.05 M, pH 7.4). The samples were excited at 450 nm and the fluorescence intensity of the samples was measured at 485 nm with slit widths of 5 nm each, on a spectrofluorimeter (RF-5301, Shimadzu). The final concentrations of ThT and insulin were 25 μM and 10.9 μM, respectively. Control samples at different time points refer to samples incubated without agitation.

2.10. Far UV circular dichroism spectroscopy

Far UV-CD spectroscopy of insulin solutions was carried out after different time periods of agitation in a quartz cuvette with a pathlength of 0.1 cm on a spectropolarimeter (J-815, Jasco) at 25 °C. The samples, including controls, were diluted five times (final protein concentration of 17.2 μM) with sodium phosphate buffer (0.05 M, pH 7.4), immediately before recording the spectra. All spectra were buffer subtracted for background correction. Spectra were

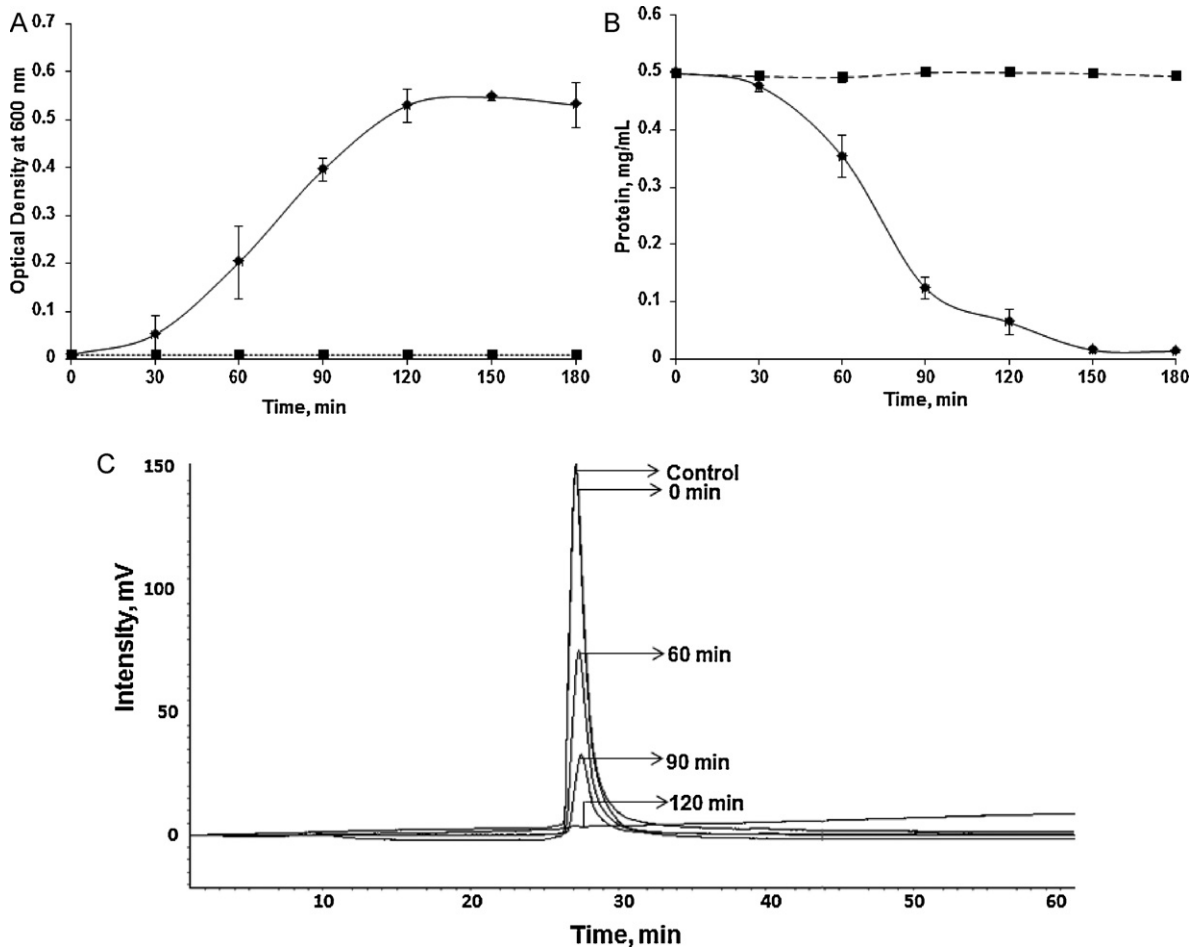

Fig. 1. Monitoring insulin aggregation at different time points. (A) The turbidity measurements of insulin samples (solid line) and control (dashed line) at different time intervals observed by light scattering at 600 nm (data represented as mean ± SD of 3 independent measurements). (B) The concentration of residual monomeric protein at different time intervals in samples (solid line) after agitation and control without agitation (dashed line) as estimated spectrophotometrically at 276 nm (data represented as mean ± SD of 3 independent measurements). (C) SEC-HPLC chromatograms representing the composition of the soluble fraction at different time intervals.

recorded in triplicate scans using a step size of 0.5 nm and a bandwidth of 1 nm. The spectra were collected between 190 nm and 250 nm. These spectra were used as input in K2D2 software for secondary structure estimation of protein samples (Perez-Iratxeta and Andrade-Navarro, 2008) and the fractions of α-helix and β-sheet contents were calculated.

2.11. Spectrofluorimetric studies

Insulin contains four tyrosine residues. Intrinsic fluorescence intensity of the agitated samples was recorded on a spectrofluorimeter (RF-5301, Shimadzu) after withdrawing aliquots at different time intervals. The samples were excited at 275 nm and the emission spectra were recorded between 290 and 360 nm with slit widths of 5 nm each. A scan of sodium phosphate buffer (0.05 M, pH 7.4) was subtracted from all the sample scans for background correction. The sample incubated for 180 min without agitation was used as the control to confirm that no change in the tertiary structure of the protein occurred during the incubation period.

3. Results and discussion

Incorporation of Zn^{2+} ions in formulations of insulin has been reported to stabilize the protein by formation of hexamers. It has been shown that when subjected to agitation *in vitro*, Zn-insulin aggregates at a much slower rate than Zn-free insulin (Costantino et al., 1994). However, the bioavailability of insulin in the hexameric form is low because of the inability of the oligomeric form to cross tissue membranes (Frokjaer and Otzen, 2005). Thus, efforts are on to stabilize Zn-free insulin. In this work, we describe the characteristics of the aggregates formed when Zn-free insulin is subjected to agitation. Insulin has been reported to aggregate at its therapeutic concentration (0.6 mM, corresponding to 3.5 mg ml^{-1}) (Brange et al., 1997). We chose the concentration of 0.5 mg ml^{-1} to work with since at this concentration, insulin has been reported to exist mostly in the monomeric and dimeric forms (Brange et al., 1997).

3.1. Aggregation of insulin

Zn^{2+} ions were removed from commercially available Zn-insulin as already described (Costantino et al., 1994). This preparation, referred to as Zn-free insulin or K-insulin (0.5 mg ml^{-1} in 0.05 M sodium phosphate buffer, pH 7.4), was subjected to agitation at 37 °C and 300 rpm to mimic the conditions of accelerated storage (Banga and Mitra, 1993). Aggregation was confirmed by increased turbidity of the solution (Fig. 1A). Under these conditions, insulin aggregated to the maximum value within 120 min. Estimation of soluble protein in the sample confirmed that all the insulin had aggregated by 120 min (Fig. 1B), which matches the results observed with turbidity measurements (Fig. 1A). The extent of aggregation was confirmed by SEC-HPLC (Fig. 1C). Elution with

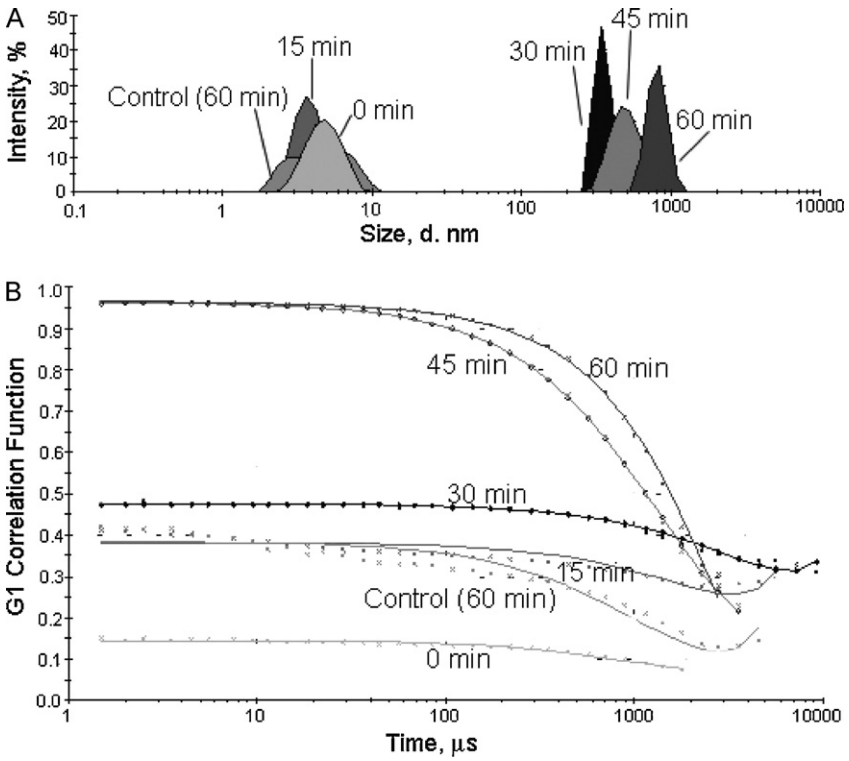

Fig. 2. Particle size analysis of insulin suspension at different intervals of agitation. (A) Dynamic light scattering of insulin suspension was measured at different time periods of agitation as a function of the particle size distribution. Control samples were incubated at 37 °C without agitation. (B) Cumulant fit graph showing correlation functions of aggregated insulin samples measured by dynamic light scattering.

sodium phosphate buffer (0.05 M, pH 7.4) resulted in continually decreasing peak area of monomeric insulin with increase in the time of agitation of protein. No additional peak was seen in the chromatogram indicating the absence of either fragmented protein or soluble aggregates under these conditions. Similar result observed earlier has been attributed to the formation of insoluble aggregates via non-covalent interactions (Sluzky et al., 1991). The transient nature of the intermediate may explain why no soluble aggregates were seen in the chromatograms. A model for agitation-induced aggregation of insulin has been proposed in which the short-lived intermediates can either go back to the monomeric form or combine with each other to form aggregates (Sluzky et al., 1991).

3.2. Particle size analysis

The size of the insulin molecule was monitored by dynamic light scattering (DLS) (Fig. 2A). The size of the molecule in the starting solution was found to be 3.76 nm (±0.28 nm) which corresponds to that of monomeric/dimeric insulin (Ahmad et al., 2005). The mean size of the insulin molecule increased with the time of agitation and after 60 min, reached a mean particle size of >700 nm. This is larger than the size that has been reported earlier (Sluzky et al., 1991). The size fractionation of the particles was in two distinct ranges (Fig. 2A), that of monomer/dimer and of aggregates. No particle with intermediate size was seen, which correlated well with the results of SEC-HPLC (Fig. 1C) and confirmed that soluble aggregates, even if formed, were short-lived transient species. The mean particle size of insulin incubated for 60 min under the same conditions but without agitation remained unchanged from that of the starting preparation (Fig. 2A). The autocorrelation function of the scattered light was fitted by cumulant analysis. The initial decay in autocorrelation function was quite fast (Fig. 2B) indicating the presence of small particles. After 15 min, the decay rate slowed down, which correlated with the increase in mean particle size observed. The mechanism of interface-mediated aggregation of insulin has been modelled as an interaction of the unstable monomeric species with the hydrophobic surface (air in this case) (Sluzky et al., 1991). The monomers, being the most unstable species, are thought to lose their conformational integrity at the interface. This results in partially unfolded monomers interacting with each other, which can shift the equilibrium either in the forward direction (leading to the formation of aggregates) or in the reverse direction (retaining the native monomeric conformation).

3.3. Analysis of aggregates

With a view to understand the nature of the aggregates formed by agitation of insulin, the aggregates were analyzed by different techniques. In order to determine whether the aggregates were formed via covalent linkages, the number of free amine groups was measured in the precipitated aggregates following agitation. As can be seen (Fig. 3), the number of free amine groups does not change in the case of the control sample (without agitation) after 60 min, indicating that covalent aggregates are not formed in the absence of agitation. In case of the agitated samples, the number of free amine groups in the aggregates undergoes a change. No variation in the number of free amine groups is seen till 30 min although DLS measurements show the formation of aggregates (Fig. 2A). This indicates that at the initial stages, aggregates are probably formed via non-covalent interactions. After 30 min, however, the number of free amine groups per mg of protein decreases in the precipitated fraction, indicating involvement of amine groups in the formation of aggregates (Fig. 3). After 120 min, the number of amine groups reaches a plateau, which can be correlated with the change in particle size seen with the DLS profile earlier. The size of the aggregates does not change (Fig. 2A) but their nature does (Fig. 3). Two distinct stages of aggregation are seen. Till 30 min, aggregation

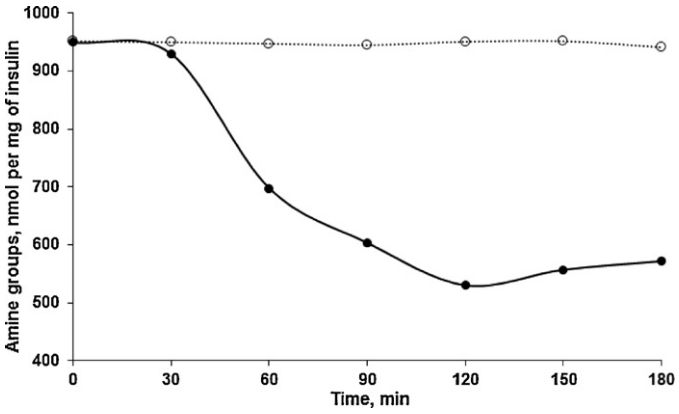

Fig. 3. Estimation of free amine groups by trinitrobenzene sulphonic acid (TNBSA) assay in agitated insulin samples (solid line) and control samples (dotted line).

Table 1
Zeta potential values (mean ± SD, $n = 3$) of insulin solution at different time intervals of agitation at 300 rpm and 37 °C.

Time of agitation (min)	Zeta potential (mV)
0	−14.5 ± 0.26
15	−16.7 ± 0.90
30	−16.8 ± 0.55
45	−13.7 ± 0.42
60	−14.1 ± 0.36

is via reversible physical interaction, and beyond 30 min, covalent linkages start playing a major role in determining the nature of aggregates. The charge/polarity of the species formed after different time periods of agitation was determined by zeta sizer and is shown in Table 1. It has been reported that zeta potential values of systems within ±30 mV have a tendency to flocculate (Domingues et al., 2009). The zeta potentials remained within the flocculation range at all time points indicating that the route to aggregation does not involve drastic changes in the pK_as of the amino acid residues involved in the formation of aggregates. Although caution needs to be exercised while using zeta potential as a parameter to surmise about the interparticle forces within protein aggregates (Nelson and Glatz, 1985), it can generally provide an idea about the changes in surface charges during coalescence of particles. The presence of a single peak in the RP-HPLC profile indicated the absence of any deamidated product in the soluble fraction (data not shown). A peak with retention time higher than that of native insulin is characteristic of desamido product (Darrington and Anderson, 1994). The absence of this peak confirmed that intramolecular cyclization had not occurred during agitation-induced aggregation of insulin. Analysis of peak areas at different time intervals confirmed the decrease in the concentration of the monomer with time (Fig. 4). Thus, denaturation via deamidation is virtually non-existent and this is consistent with literature reports (Gryniewicz and Kauffman, 2008).

Another mechanism by which covalent aggregates can be formed is via disulphide bond formation/exchange. Insulin has three (one intra- and two inter-chain) disulphide bonds and no free Cys residues. Thus, new disulphide linkages, if any, will be formed when a thiolate anion in an existing disulphide bond carries out a nucleophilic attack on another disulphide linkage (Costantino et al., 1994). Since the formation of a thiolate anion is catalyzed by hydroxide ions, insulin solutions prepared by dissolving the protein in alkaline solution are more prone to aggregation via disulphide scrambling than those that are pre-dissolved in acidic solutions (Costantino et al., 1994). In accordance with this, we could not detect the presence of any free thiol group in the aggregates isolated after different time intervals of agitation (data not shown), as determined by Ellman's reagent (Riddles et al., 1983).

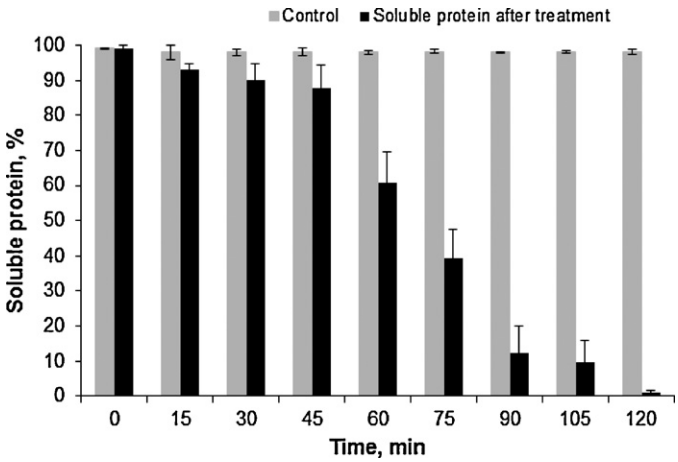

Fig. 4. The concentration of residual monomeric protein at different time intervals after agitation was estimated as a function of their peak areas following RP-HPLC (data represented as mean ± SD of 3 independent measurements). 100% refers to the amount of insulin initially subjected to agitation.

Next, we subjected the aggregates to native polyacrylamide gel electrophoresis (PAGE) after dissolving the aggregates in different denaturing and reducing reagents (Fig. 5A). The aggregates suspended in sodium phosphate buffer (0.05 M, pH 7.4) did not dissolve at all and were thus unable to enter the gel. As expected from the data of thiol group estimation, aggregates suspended in 0.01 M dithiothreitol (DTT) remained insoluble, confirming that the covalent bonds involved in the formation of aggregates are of non-disulphide nature. Similar results were obtained with denaturing (SDS) PAGE carried out under reducing conditions (data not shown). Maximum amount of dissolution occurred in 8 M urea, indicating that at least a fraction of the aggregates formed is of non-covalent nature. This is similar to the properties of the aggregates that are reported to form when insulin solution aggregates in a water/oil emulsion (Taluja and Bae, 2007). Taken together with the data from free amine group analysis, it can be concluded that the insulin aggregates formed at the air–water interface are formed via both non-covalent as well as non-reducible covalent interactions involving amine groups. Non-disulphide-mediated covalent aggregation of insulin in the solid state is reported to be initiated via deamidation of Asn_{A21} followed by dimerization of Asp_{A21}-Phe_{B1} and Asp_{A21}-Gly_{A1} (Lai and Topp, 1999). In our case however, no evidence for the formation of any deamidated product in the soluble fraction by RP-HPLC could be detected (data not shown). Thus, covalent aggregation probably involved residues other than the above. One potential candidate is the ε-amino group of Lys_{B29} present on the hydrophobic face of the protein. The interaction of Lys_{B29} with Pro_{B28} has been reported to be the nucleus for self-association of insulin and is the basis of designing Lispro, the first engineered rapid-acting insulin analogue (Vajo and Duckworth, 2000). In this, the aggregation propensity of insulin is diminished by interchanging the positions of Lys and Pro.

MALDI-TOF mass spectra of control insulin, supernatant obtained after aggregation (soluble fraction) and supernatant obtained after urea-dissolution of insulin aggregate (urea-solubilized fraction) showed identical profiles, with $[M+H]^{+1}$ peak at 5743 (Fig. 5B). This confirmed that no soluble aggregates were seen even within 60 min of agitation. The solubilisation of a higher amount of protein in the presence of 8 M urea (Fig. 5A) and the appearance of a single peak show that non-covalent interactions are involved in the formation of aggregates following agitation. The presence of residual pellet after centrifugation of

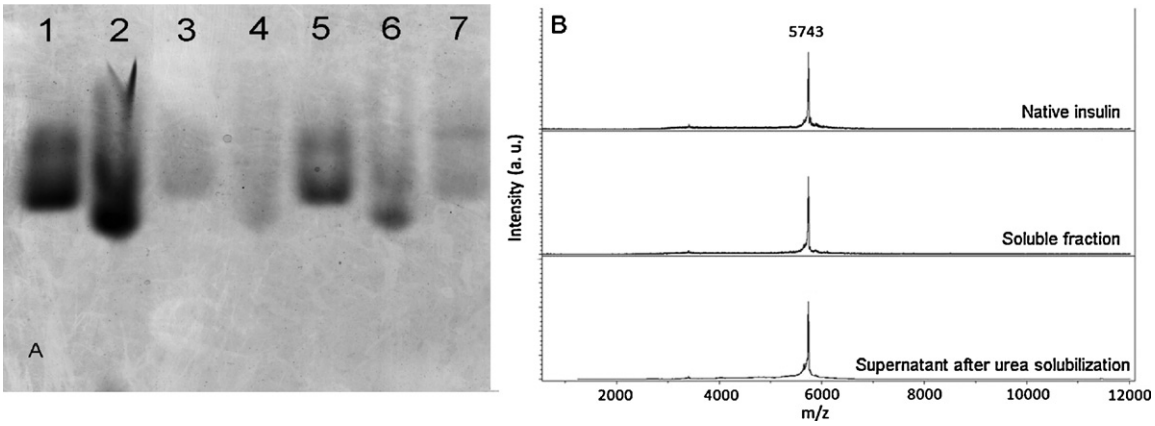

Fig. 5. Analysis of insulin samples after agitation. (A) Native polyacrylamide gel (15% crosslinked) for insulin aggregates, 20 μg each resuspended in buffer and denaturing reagents. Lanes 1, 3, 5 and 7 show insulin samples analyzed under reducing conditions while lanes 2, 4 and 6 show insulin samples analyzed under non-reducing conditions. Lanes 1 and 2: control insulin; lanes 3 and 4: insulin aggregates resuspended in sodium phosphate buffer (0.05 M, pH 7.4); lanes 5 and 6: insulin aggregates resuspended in 8 M urea; lane 7: insulin aggregates resuspended in 10 mM DTT. The gel was silver stained for visualization of protein bands. (B) MALDI-TOF mass spectra of control insulin, soluble fraction of sample treated for 60 min and soluble fraction of 60 min aggregates resuspended in 6 M urea and dialyzed against deionised water obtained after centrifugation.

the urea-suspended sample confirmed that interactions other than non-covalent forces are involved in the formation of insulin aggregates.

3.4. Spectroscopic analysis of aggregates

Far UV-CD spectroscopy was carried out to study the changes in the secondary structure of insulin following agitation-induced aggregation. Starting insulin sample exhibited a spectrum characteristic of the protein with minima at 208 and 222 nm (inset of Fig. 6A) (Ahmad et al., 2005). With increase in the time of agitation, the ellipticity at these wavelengths decreased, reflecting the loss of α-helix content of the protein (Fig. 6A). After 90 min, the minima at 208 nm had disappeared completely showing the loss of α-helix. The aggregates formed after different time intervals were incubated with Thioflavin T (ThT) and the emission intensity of ThT was measured (Fig. 6B). Agitation induces fibrillation of insulin (Nielsen et al., 2001b) and binding of ThT with amyloid fibrils containing crossed β-sheet structure exhibits a drastic enhancement in its emission maxima. The kinetics of ThT fluorescence showed a lag time of 30 min after which the fluorescence intensity of the dye increased significantly, reaching a plateau after 90 min (Fig. 6B). Comparison of the graph of fluorescence intensity of ThT (Fig. 6B) with change in the secondary structure (Fig. 6A) at different time intervals after agitation showed that there are two distinct stages of aggregate formation. In the first stage, the α-helix content of the aggregates as well as their ThT fluorescence remain high. This is referred to as "sulphate-type" aggregate and has been seen in the case of glucagon in the presence of sulphate ions in the solvent (Pedersen and Otzen, 2008). In the second stage, the β-sheet content of the aggregates increases while their ThT fluorescence remains unchanged. This is referred to as "Type A" aggregate and is normally seen at high protein concentrations (Pedersen and Otzen, 2008). More than one type of aggregate can occur simultaneously in solution and the relative proportion of each is decided by the concentration of solute and ionic strength and pH of the solvent (Pedersen and Otzen, 2008). This can also be correlated with the kinetic model of insulin aggregation (Sluzky et al., 1991) which refers to biphasic kinetics of aggregation following agitation. With ^{13}C-NMR spectroscopy (Petkova et al., 2005) and time-lapsed atomic force microscopy (Loksztejn and Dzwolak, 2010), it has recently been shown that the nature of amyloid aggregates formed after agitation of protein samples varied with the conditions of agitation. Two distinct stages, 'early' and 'delayed', were recognized in case of agitation-induced aggregation of insulin at 60 °C (Loksztejn and Dzwolak, 2010). It was proposed that the initial amyloid structure was formed because of the propensity of the protein molecule to form optically active macrostructure even in the absence of agitation. However, retention of these amyloidogenic structures required continuous agitation of insulin. Thus, a two-stage nucleation process has been proposed (Loksztejn and Dzwolak, 2010; Jansen et al., 2005), which is similar to the hypothesis proposed earlier (Sluzky et al., 1991). In the first ('early') stage, nucleation leads to the formation of the amyloid skeleton. This is followed by a phase ('delayed' stage) in which continuous agitation results in the formation of amyloid macrostructures. Conditions employed in the 'early' stage decide the nature of the aggregates formed in the 'delayed' stage. Fibrils formed by insulin in quiescent solutions do not lead to the formation of optically active structures even after agitation whereas vigorous agitation in the first stage leads to the formation of amyloid aggregates with negative Cotton effect (Loksztejn and Dzwolak, 2010). The presence of ThT-positive filaments within 30 min of agitation at 37 °C (Fig. 6B) suggests that the amyloid fibrils formed at this ('early') stage offer a scaffold on which the chiral superstructure is built up.

Intrinsic fluorescence intensity of agitated insulin samples was monitored at different time periods during agitation to decipher the changes occurring around the fluorophore and thus, in the overall folding architecture of the insulin molecule. When excited at 275 nm, insulin aggregates, formed after agitation, did not show any change in the wavelength of maximum emission intensity [$\lambda_{max(emission)}$] from that of the control protein sample (λ_{max} = 303 nm in both cases) (Fig. 7). Thus the microenvironment around the fluorophores does not undergo any significant change in polarity upon aggregation. This correlates well with the earlier observation that non-covalent forces play a role in the initial formation of aggregates, keeping the solvent accessibility of the fluorophore unaltered. The intrinsic fluorescence of insulin is because of the presence of four tyrosine residues (Bekard and Dunstan, 2009). A steady decrease in the emission intensity of the protein at 303 nm with increase in time of agitation could also indicate the removal of insoluble protein from solution. However, within the time period of measurement, the aggregated protein remains uniformly suspended in the cuvette. Thus, the pattern of spectra obtained with gradually decreasing fluorescence intensity indicates the increased contribution of scattering and the burial of the

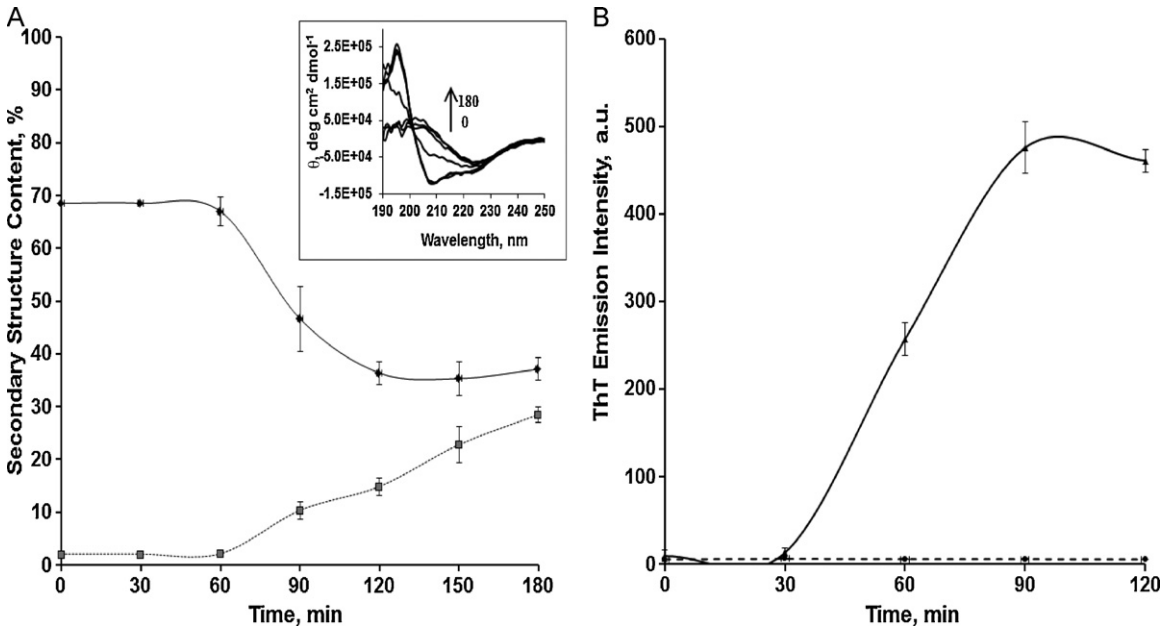

Fig. 6. Analysis of aggregates formed after agitation of insulin. (A) Secondary structure content of insulin after different time intervals of agitation as calculated by using K2D2 software for secondary structure calculation of protein (Perez-Iratxeta and Andrade-Navarro, 2008). The α-helical content is represented by circles and β-sheet content by squares (data represented as mean ± SD of 3 independent measurements). Inset shows far-UV CD spectra of samples showing residual secondary structure of insulin at different time intervals of agitation. Numbers denote the time (from 0, 30, 60, 90, 120, 150 to 180 min) of agitation after which the spectra were recorded. (B) Thioflavin T fluorescence emission intensity recorded in the presence of insulin samples for different time periods for agitated samples (solid line) and for control samples (dotted line) (data represented as mean ± SD of 3 independent measurements).

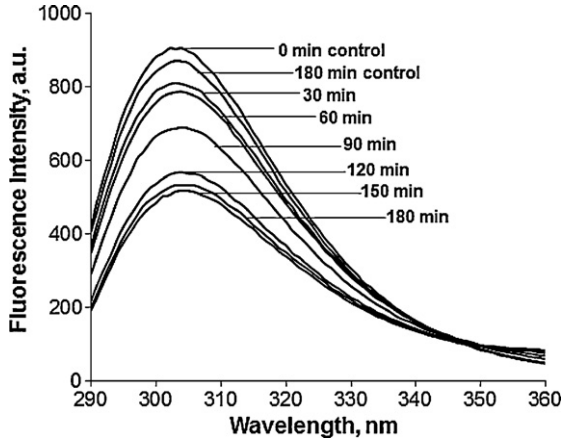

Fig. 7. Intrinsic fluorescence spectra of insulin samples agitated for different time periods.

fluorophores within the aggregates, making them less available to be excited at 275 nm.

4. Conclusion

The two-stage model of agitation-induced aggregation of insulin has been validated by the present study. The initial (early) stage involves non-covalent interaction between monomeric/dimeric molecules. This is reflected in the negligible change in the secondary structure till 60 min. This is followed by the 'delayed' stage where neighbouring protein molecules start interacting via non-disulphide-mediated covalent interactions leading to a significant change in the tertiary fold as well as the optical properties of the insulin aggregates, reflected in the far UV-CD spectra and ThT fluorescence measurements.

The study emphasises the effect of agitation on protein solutions, which is an important stress factor for liquid protein formulations. More specifically, it was examined that not only non-covalent interactions are involved but covalent bonds also form between the partially unfolded protein molecules. Thus, formation of irreversible aggregates occurs which leads to problems like immunogenicity (Fineberg et al., 2007; Sauerborn et al., 2010) and low bioavailability (Pezron et al., 2002). Characterization of the aggregates may help in finding out new candidates that can interact with the native protein to reduce the interactions that lead to aggregate formation.

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