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Improving the stability of the EC1 domain of E-cadherin by thiol alkylation of the cysteine residue

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ABSTRACT

The objective of this work was to improve chemical and physical stability of the EC1 protein derived from the extracellular domain of E-cadherin. In solution, the EC1 protein has been shown to form a covalent dimer via a disulfide bond formation followed by physical aggregation and precipitation. To improve solution stability of the EC1 protein, the thiol group of the Cys13 residue in EC1 was alkylated with iodoacetate, iodoacetamide, and maleimide-PEG-5000 to produce thioether derivatives called EC1-IA, EC1-IN, and EC1-PEG. The physical and chemical stabilities of the EC1 derivatives and the parent EC1 were evaluated at various pHs (3.0, 7.0, and 9.0) and temperatures (0, 3, 70 °C). The structural characteristics of each molecule were analyzed by circular dichroism (CD) and fluorescence spectroscopy and the derivatives have similar secondary structure as the parent EC1 protein at pH 7.0. Both EC1-IN and EC1-PEG derivatives showed better chemical and physical stability profiles than did the parent EC1 at pH 7.0. EC1-PEG had the best stability profile compared to EC1-IN and EC1 in solution under various conditions.

1. Introduction

E-cadherin is a calcium-binding glycoprotein found at the adherens junction of the intercellular junctions of the intestinal mucosa (Zheng et al., 2006). It is one of the important proteins that mediate cell–cell adhesion at the intercellular junctions. Modulation of cadherin interactions at the intercellular junctions by cadherin peptides improves the delivery of paracellular marker molecules (i.e., ^{14}C mannitol) and anticancer drugs (i.e., 3H-daunomycin) through the blood–brain barrier (Kiptoo et al., 2011). The EC1 domain of E-cadherin has an important role in the selectivity of homophilic E-cadherin interactions as well as in the heterophilic interaction of E-cadherin with $\alpha_E\beta_7$ integrin to create cell–cell adhesion (Pokutta et al., 2008; Pokutta and Weis, 2007; Shiraishi et al., 2005; Zheng et al., 2006). To study the biological function of the EC1 domain to modulate cell–cell adhesion at the intercellular junctions of the intestinal mucosa, we expressed the recombinant human EC1 protein. Unfortunately, the EC1 protein is unstable during storage (i.e., 4 weeks at 4 °C) after purification. It produces a covalent dimer (EC1-s-s-EC1) via an intermolecular disulfide bond between the only Cys residue (Cys13) in the monomer, and the formation of a covalent dimer induces the generation of oligomers and precipitation (Makagiansar et al., 2002b; Trivedi et al., 2009). The presence of the covalent dimer and physical oligomers has been characterized using SDS-PAGE and size-exclusion chromatography (SEC) as well as by monitoring changes in its far UV circular dichroism (CD) spectra and intrinsic fluorescence emission spectra after storage at 4 °C. The presence of precipitates prevents long-term storage and interferes with the study of the biological activity of EC1 as an inhibitor of cell–cell adhesion. Therefore, it is necessary to find a suitable method to improve the storage stability of this protein for its use in future biological activity evaluations in inhibiting cell–cell adhesion.

Accelerated stability studies at different pH values (3.0, 7.0, and 9.0) and temperatures (37 °C and 70 °C) have shown that the EC1 protein also undergoes hydrolysis at the D93–P94 peptide bond (Trivedi et al., 2009). Molecular dynamics simulation studies have indicated that the covalent dimer has a higher dynamic mobility than the monomer, thereby contributing to the instability of the protein (Trivedi et al., 2009). The increased flexibility of the EC1 dimer could explain the susceptibility of the D93–P94 peptide bond to hydrolysis and physical oligomerization. It is interesting to find that chemical and physical degradation can be inhibited by addition of dithiothreitol (DTT) as a reducing agent to prevent the formation of a covalent dimer. However, the presence of reducing agents (i.e., DTT or glutathione) would be undesirable during biological activity studies (i.e., inhibition of cell–cell adhesion) of EC1 since they may affect the results of the cell adhesion assays. Thus, an alternative

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method is needed to stabilize the monomeric form of the EC1 protein so it can be evaluated in biological assays and stored for long periods of time.

To eliminate the possibility of covalent dimerization of the EC1 protein, the thiol group of the Cys13 residue in EC1 was alkylated to produce thioether derivatives called EC1-IA, EC1-IN, and EC1-PEG (Fig. 1). To produce EC1-IA and EC1-IN, the thiol group of EC1 was reacted with iodoacetic acid and iodoacetamide, respectively; this method has been extensively applied to modify protein (Dickens, 1933; Smythe, 1936). Next, the EC1 protein was derivatized with polyethylene glycol-5000 (PEG5000) to prepare EC1-PEG by reacting the Cys13 thiol group with the maleimide group on the PEG5000 (Bell et al., 2008; Colonna et al., 2008). The physical and chemical stabilities of the EC1 derivatives were compared to those of the parent EC1 and evaluated at various pHs (3.0, 7.0, and 9.0) and temperatures (0, 37, 70 °C). The structural characteristics of EC1 derivatives during the stability studies were analyzed by circular dichroism (CD) and fluorescence spectroscopy (Trivedi et al., 2009). The stability of both EC1-IN and EC1-PEG derivatives was better than that of the parent EC1 at pH 7.0. Compared to EC1-IN and EC1, the EC1-PEG had the best stability profile under various conditions.

2. Experimental methods

2.1. Production of EC1-IA and EC1-IN

The EC1 protein (MW = 11,628 Da) was expressed in *E. coli* and purified in the presence of β-mercaptoethanol (BME) (Makagiansar et al., 2002a,b). The EC1 protein (0.2 mg/mL) was reacted in the dark with iodoacetate or iodoacetamide (1.0 mM) for 4 h at pH 8.0 in a buffer containing 0.02 mM BME, 20 mM Tris, and 4.0 M guanidine hydrochloride (Gdn.HCl). The completion of the reaction was monitored and confirmed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry with time-of-flight (TOF) detection. The alkylated EC1 (EC1-IA and EC1-IN) was refolded by employing a stepwise dilution method in which the concentration of Gdn.HCl was reduced from 4.0 M to 0.5 M with a 0.5 M decrease in each dilution step. The dilution step consists of the addition of an appropriate volume of a buffer containing 20 mM Tris at pH 8.0, followed by incubation at 4 °C for 8 h. When the Gdn.HCl concentration reached 0.5 M, dialysis was performed with an 8-kDa membrane to remove the Gdn.HCl, BME, and the unreacted iodoacetate or iodoacetamide. Far UV CD spectroscopy was used to determine the success of protein refolding.

2.2. Production of EC1-PEG

Maleimide-PEG (250 mg, MW = 5000 Da, Sigma Chemical Co., St. Louis) was added to 50 mL of 20 mM Tris buffer at pH 8.0 containing 0.2 mg/mL of EC1 and 0.2 mM DTT. The reaction mixture was incubated for 2 h at 22 °C followed by injection of 50 μL of the solution to MALDI-TOF MS analysis. The remaining solution was concentrated to 10 mL by passing it through a 10,000 Da cutoff membrane in an Amicon centrifuge tube and centrifuging at 4000 × g. The concentrated solution was stored at 4 °C until it was purified by SEC.

Three mL of the above concentrated solution was injected into a Superdex 200 size-exclusion column with a mobile phase of 20 mM Tris buffer at pH 8.0 and a flow rate of 0.5 mL/min. The EC1-PEG conjugate, parent EC1, and the maleimide-PEG were detected as separate peaks in the SEC chromatogram by their UV absorbance at 220 or 280 nm, and the yield of EC1-PEG was 75%. The column volume was 150 mL, and the volume of each collected fraction was 3.0 mL. The EC1-PEG fractions were pooled and concentrated to 7.5 mL. The concentration of EC1-PEG in this final solution was equal to 86 μM of EC1. Fifty μL of this EC1-PEG solution was analyzed by MALDI-TOF. The remaining EC1-PEG was used for chemical stability studies using high performance liquid chromatography (HPLC) analysis and structural studies using far CD spectroscopy and intrinsic fluorescence emission spectroscopy.

2.3. Chemical stability studies of EC1-IN and EC1-PEG

The EC1-IN and EC1-PEG proteins were dialyzed into pH 3.0, 7.0, and 9.0 buffers and incubated at a concentration of 86 μM in sealed vials at 4, 37, and 70 °C for 4 h (Trivedi et al., 2009). The buffers used were 100 mM phosphate for pH 3.0, 50 mM phosphate for pH 7.0, and 100 mM borate containing 0.08 M NaCl for pH 9.0. Samples were drawn at 0, 0.5, 1.0, 2.0, and 4.0 h and immediately stored at −70 °C until subsequent HPLC analysis was performed. The HPLC was equipped with a C18 column (Varian Microsorb; pore-size: 300 Å; dimensions 250 mm × 4.6 mm) for separation of the analytes. The protein solution (25 μL) was injected into the column and eluted at a rate of 1.0 mL/min. A gradient elution method incorporating solvent A (94.9% double distilled water, 5% acetonitrile (ACN), and 0.1% TFA) and solvent B (100% ACN) was used. The gradient elution followed the sequence 0–45% B from 0.0 to 2.0 min, 45–51% B from 2.0 to 12.0 min, at 51% B from 12.0 to 17.0 min, 51–100% B from 17.0 to 19.0 min, at 100% B from 19.0 to 21.0 min, and 100% B back down to 0% B from 21.0 to 22.0 min. A Varian Prostar UV detector at a wavelength of 220 nm was used to detect the proteins eluted from the column. MALDI-TOF MS analysis was used to determine the molecular weight of any new peaks observed on the HPLC chromatogram.

2.4. Physical stability studies

Physical stability studies of EC1-IN and EC1-PEG were performed by monitoring the far UV CD spectra and the intrinsic fluorescence emission spectra at days 0, 14, and 28 of incubation at 4 °C and pH 3.0, 7.0 and 9.0. The concentrations and buffers used were the same as described in Section 2.3.

2.4.1. Far UV CD studies

The secondary structural changes of the EC1-IN or EC1-PEG protein were analyzed by far UV CD spectroscopy with a Jasco spectropolarimeter (J-720) equipped with a Peltier temperature controller by loading 300 μL of the protein into a sealed CD cuvette with 0.1 cm pathlength (Derrick et al., 2004; Zheng et al., 2009a,b). The protein CD spectrum was measured at 10 °C between 200 and 250 nm. Triplicate spectra were obtained for EC1-IN or EC1-PEG, and the contribution of the buffer was eliminated by subtracting the blank spectrum (containing buffer alone) from the spectrum of protein in the same buffer. The same samples were also used for the thermal unfolding studies by recording their CD spectra at every 5 °C increase in temperature. The temperature of the cuvette holder was increased from 10 °C to 65 °C at a rate of 15 °C/h. The samples were equilibrated at the target temperature for 300 s before obtaining their spectra. The entire procedure was repeated after 14 and 28 days for samples stored at pH 3.0, 7.0, and 9.0 at 4 °C. The ellipticity at 218 nm (used to monitor changes in the β-sheet content) was plotted against the corresponding temperature, and a sigmoidal function was used to fit the thermal unfolding curves using Origin 7.0 software. The midpoint of each transition in the sigmoidal fit was defined as the thermal unfolding temperature (T_m).

2.4.2. Intrinsic fluorescence emission studies

Changes in the tertiary structure of EC1-IN and EC1-PEG after 14 and 28 days incubation at 4 °C and different pH values (3.0, 7.0, or 9.0) were monitored using fluorescence spectroscopy and compared to the emission profile on day 0. For each experiment,

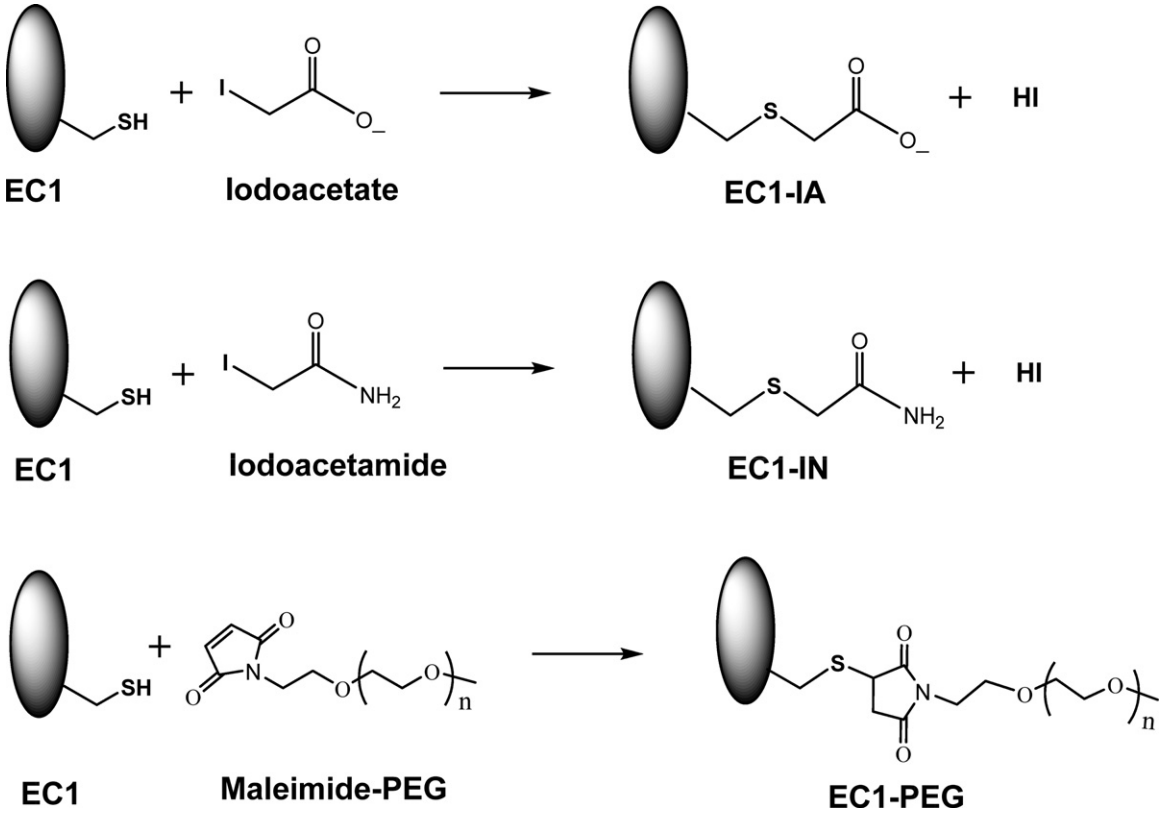

Fig. 1. The alkylation reaction of the thiol group on the Cys13 residue of EC1 with iodoacetate and iodoacetamide produces the EC1 derivatives EC1-IA and EC1-IN, respectively. The alkylation of the thiol group with maleimide-PEG-5000 generates EC1-PEG molecule.

90 μL of the protein (86 μM) was diluted to 0.9 mL with the appropriate buffer and transferred to a quartz fluorescence cuvette. The protein was excited at 295 nm (>95% Trp emission), and the fluorescence emission was observed between 305 and 405 nm using a PTI QuantaMaster spectrofluorometer (Rexroad et al., 2003; Zheng et al., 2009a,b). Emission was set between 500,000 and 1,000,000 counts/s by adjusting the excitation and emission slits. The emission spectra were obtained in triplicate, and the blank spectrum was subtracted from each of them. Thermal unfolding of EC1-IN and EC1-PEG was studied at pH 3.0, 7.0, and 9.0 by increasing the temperature of the cuvette chamber from 10 °C to 87.5 °C at a rate of 15 °C/h; the emission spectra were recorded at every 2.5 °C increase in temperature. Each fluorescence emission spectrum obtained was fitted to an extreme asymmetric peak function using the non-linear curve-fitting wizard of Origin 7.0 software to obtain the wavelength of maximum emission. After subtracting the blank emission signal from each spectrum, the wavelength of maximum emission at each temperature point was plotted against the corresponding temperature. This plot, which provided the thermal unfolding profile of the protein, was then fitted to a sigmoidal function using Origin 7.0 software.

3. Results

3.1. Alkylation of the thiol group of EC1

The alkylation of EC1 with iodoacetate and iodoacetamide (Fig. 1) produced the desired EC1-IA and EC1-IN in quantitative yields with one band in an SDS PAGE gel and a single peak on a reversed-phase HPLC C18 column. MALDI mass spectra showed the addition of acetate and acetamide groups in EC1-IA (MW = 11,686.6 Da) and EC1-IN (MW = 11,685.3 Da), respectively. The reaction between Cys thiol of EC1 and maleimide-PEG produced EC1-PEG in 75% yield, and the desired EC1-PEG was separated from the EC1 and PEG by SEC. Using a C18 reversed-phase column, EC1-PEG showed a single peak at 8.1 min compared to EC1 at 8.4 min and maleimide-PEG at 7.9 min. The MALDI spectrum of EC1-PEG showed a broad peak centered around MW of 16,927 due to a distribution of molecular weights of the PEG group around 5000 Da.

The CD spectra of EC1-IA and EC1-IN were compared with that of EC1. The EC1-IN protein has a spectrum identical to that of EC1 at pH 7.0 with a minimum at 216 nm and maxima at 235 and 205 nm (Fig. 2). However, the CD spectra of EC1-IA at various pH values showed that this protein was not properly folded (data not shown); thus, it was not investigated further for the stability studies. The

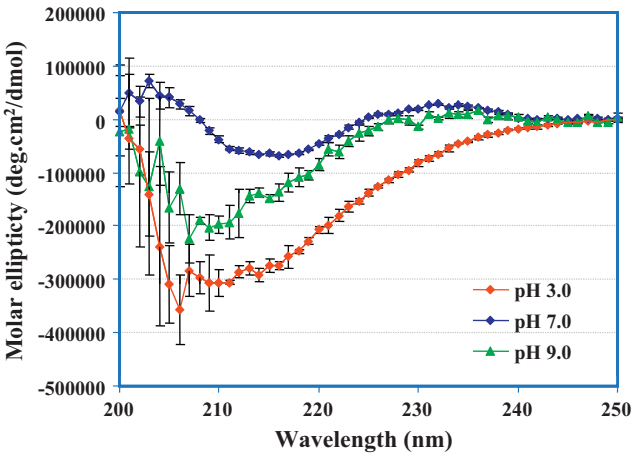

Fig. 2. The CD spectra of EC1-IN in buffered solutions at pH 3.0, 7.0, and 9.0. The secondary structure of EC1-IN is affected by the pH of the solution.

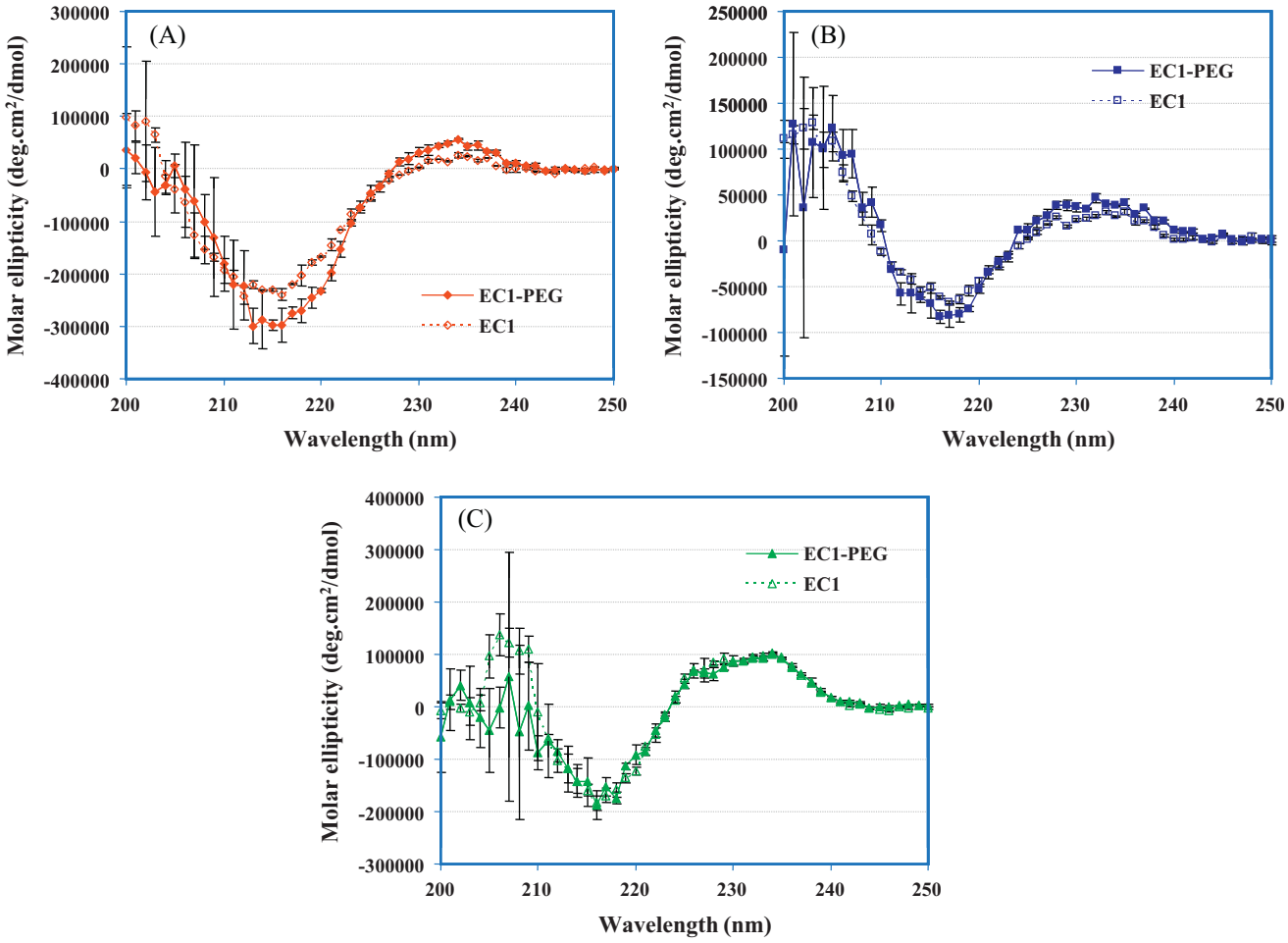

Fig. 3. Comparisons of the secondary structures of EC1 and EC1-PEG using far UV CD at pH: (A) 3.0, (B) 7.0, and (C) 9.0. The spectrum of EC1-PEG is slightly different than that of EC1 at pH 3.0. However, the CD spectra of EC1-PEG at pH 7.0 and 9.0 are similar to those from EC1, suggesting that EC1-PEG and EC1 have similar secondary structures at pH 7.0 and 9.0.

secondary structure of EC1-IN is sensitive to pH changes, indicated by the CD spectra of EC1-IN at pH 3.0, 7.0, and 9.0 (Fig. 2). The minimum at 216 nm at pH 7.0 shifted to 210 nm at pH 3.0 and 9.0. Furthermore, the spectra at pH 3.0 and 9.0 lack the maxima at 205 and 235 nm. These results indicate that EC1-IN has a secondary structure similar to that of the parent EC1 at pH 7.0, but not at pH 3.0 and 9.0.

The CD spectra of EC1-PEG were compared to those of EC1 at pH 3.0, 7.0, and 9.0. At pH 3.0, a minor difference was observed between the spectra of the two proteins (Fig. 3A) in which the spectrum of EC1-PEG had more pronounced positive and negative peaks at 235 nm and 216 nm, respectively. There was no difference between the spectra of EC1-PEG and EC1 at pH 7.0 (Fig. 3B) and 9.0 (Fig. 3C), suggesting that the PEGylated protein maintains its conformation.

3.2. Chemical stability comparison of EC1, EC1-IN, and EC1-PEG

The chemical stability of the EC1-IN protein was monitored by HPLC after incubation for 4 h at 4 °C and pH 3.0, 7.0, and 9.0. Under these conditions, there was no observable decrease in the chromatographic peak of the EC1-IN protein and no appearance of a new peak for any degradation product (data not shown). These results indicate that the EC1-IN protein is stable upon incubation for 4 h at 4 °C at all three pH values.

The EC1-IN protein was also chemically stable for 4-h incubation at 37 °C and pH 3.0, 7.0, and 9.0 (Fig. 4A) with no significant change in the area under the peak of EC1-IN and no new peaks representing the degradation products. This result is in contrast to our previous studies on EC1 in which EC1 was unstable during 4-h incubation at pH 7.0 and 9.0 at 37 °C with the observed Asp93–Pro94 peptide bond hydrolysis product (Trivedi et al., 2009). These results imply that the EC1-IN protein is more stable to hydrolytic cleavage than is the parent EC1 protein at 37 °C.

The EC1-IN protein was also stable for 4 h at pH 7.0 and 9.0 at 70 °C (Fig. 4B), which is more stable than the parent EC1 under these conditions. Previously, the EC1 protein was shown to undergo Asp93–Pro94 peptide bond hydrolysis and precipitation under similar conditions (Trivedi et al., 2009). Under pH 3.0 and 4-h incubation at 70 °C, however, a significant decrease in the amount of EC1-IN (to 22%) was observed, followed by the appearance a new peak with MW = 10,443.5 Da corresponding to the hydrolysis product at the Asp93–Pro94 peptide bond (Fig. 4B). Moreover, at pH 3.0, the increase in the peak area of the hydrolysis product did not correspond quantitatively to the decrease in the peak area of the EC1-IN protein; this was due to the aggregation that leads to precipitation of the protein.

The chemical stability of EC1-PEG at 70 °C was compared with that of EC1-IN and EC1 by incubation at pH 3.0, 7.0, and 9.0 for 4 h (Fig. 5). At all three pH values studied, the amount of EC1-PEG did not change substantially after incubation for 4 h at 70 °C. Moreover, no new peaks appeared in the chromatograms. This suggests that, at high temperature, EC1-PEG is stable to precipitation and to chemical degradation reactions such as peptide bond hydrolysis. In contrast, the amount of EC1 remaining in solution was the lowest

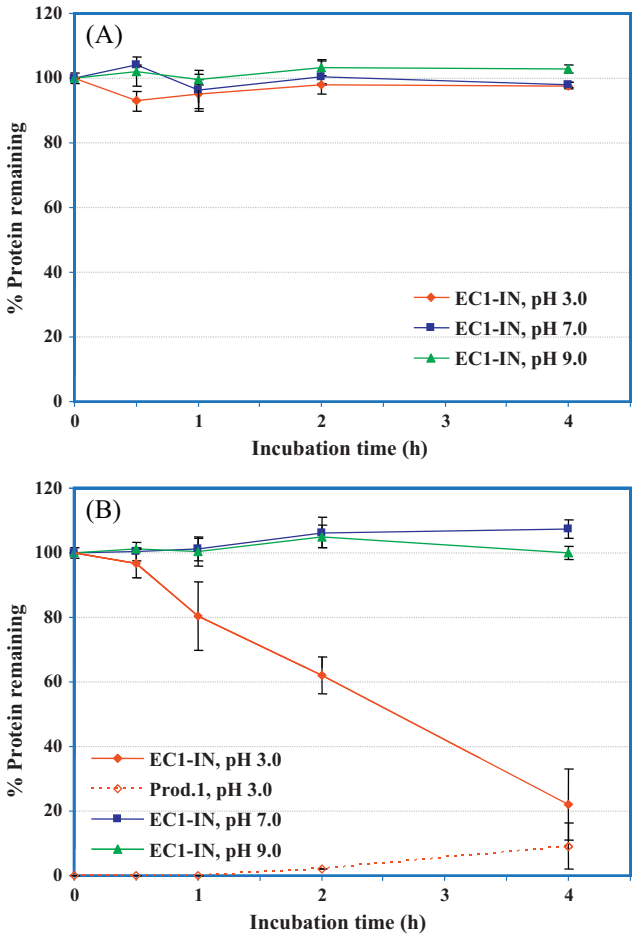

Fig. 4. The chemical stability profiles of EC1-IN after incubation for 4 h at pH 3.0, 7.0, and 9.0 at: (A) 37 °C and (B) 70 °C. At 37 °C, EC1-IN does not undergo any chemical degradation or precipitation reaction at pH 3.0, 7.0, or 9.0. At 70 °C, EC1-IN shows no peptide bond hydrolysis or precipitation at pH 7.0 or 9.0; however, EC1-IN undergoes peptide bond hydrolysis as well as precipitation at pH 3.0. The product of hydrolysis was identified as the N-terminal fragment G1–D93, a product of hydrolysis at the D93–P94 peptide bond.

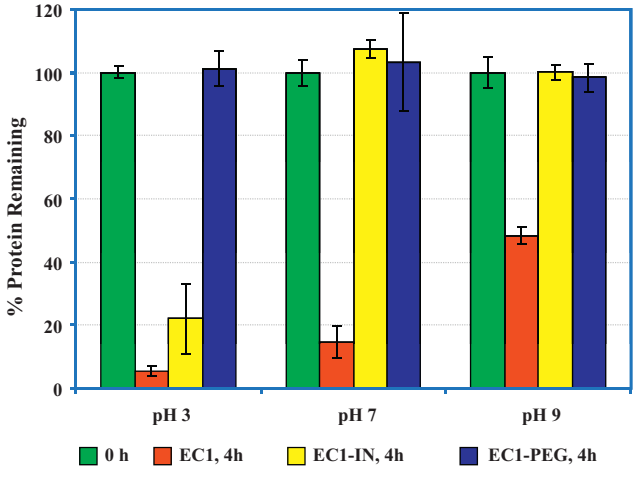

Fig. 5. A comparison of the chemical stability of EC1, EC1-IN, and EC1-PEG at pH 3.0, 7.0, and 9.0 after 4-h incubation at 70 °C. It is clear that EC1-PEG is the most stable molecule at all pH values. EC-IN is stable at pH 7.0 and 9.0 but not at pH 3.0. EC1 is the least stable molecule at all pH values compared to EC1-PEG and EC1-IN.

upon incubation at pH 3.0, followed by incubation at pH 7.0 and pH 9.0. The EC1-IN protein was also more stable than the EC1 protein at pH 7.0 and 9.0; however, it was unstable at pH 3.0.

3.3. Physical stability comparison of EC1, EC1-IN, and EC1-PEG

3.3.1. Secondary structure studies of EC1 and EC1-IN using CD spectroscopy

The effect on its physical stability of incubating EC1-IN at pH 3.0, 7.0 and 9.0 at 4 °C for up to 28 days was studied by observing changes in its CD spectra and comparing the data to the CD spectra of the parent EC1 protein. At pH 3.0, the CD spectra of the EC1-IN protein did not change after incubation for 14 days (Fig. 6A). The CD spectra of EC1, however, changed from day 0 to day 14 with a shift in the minimum at 216 nm and disappearance of the maxima at 235 and 205 nm (Fig. 6A), suggesting an increase in β-sheet character that could be due to the formation of oligomers. The melting curve of EC1-IN was compared to that of EC1 by monitoring the change in the CD signal at 218 nm (Fig. 7A). At pH 3.0, there is a very small difference in the melting curve of EC1-IN after 14-day incubation, whereas a dramatic change is detected in the melting curve of EC1 after 14-day incubation. These data imply that EC1-IN does not undergo changes such as covalent dimerization or physical oligomerization when incubated at 4 °C for 14 days as does EC1.

At pH 7.0, the CD spectra of EC1-IN protein did not change upon incubation for 0, 14, and 28 days at 4 °C (Fig. 6B). On the other hand, incubation of the EC1 protein under the same conditions produced changes in the CD spectra after 28 days (data not shown). The melting curves of EC1-IN after incubation for 0, 14, and 28 days did not change, but the melting curves of the EC1 protein showed dramatic changes (Fig. 7B), suggesting that EC1-IN did not undergo physical degradation upon incubation.

At pH 9.0, CD spectra of the EC1-IN protein after 14 and 28 days incubation showed a dramatic shift of the minimum from 210 nm to 202 nm, indicating a change in the secondary structure upon incubation (Fig. 6C). Conversely, the CD spectrum of EC1 did not change after incubation for 28 days (data not shown). In general, the melting curve profiles of EC1-IN were different from those of EC1 at different days. Although it was not dramatic, the melting curves of both EC1-IN and EC1 showed some change after 28 days of incubation (Fig. 7C).

3.3.2. Thermal unfolding properties of EC1-PEG

To evaluate the effect of PEGylation in EC1, thermal unfolding profiles from 10 °C to 65 °C of EC1-PEG were compared to that of EC1 at pH 3.0, 7.0, and 9.0 (Fig. 8). The data obtained for the thermal unfolding transitions were fitted to a sigmoidal function, and the mid-point of each fit was defined as the thermal unfolding temperature (T_m). The T_m values of EC1-PEG and EC1 at various pH values were compared. It is clear that the EC1-PEG has a higher T_m than EC1 at all pH values studied, indicating that alkylation of EC1 with PEG group improves the thermal stability of EC1. Both proteins have the highest T_m value at pH 7.0.

3.3.3. Tertiary structure properties of EC1-IN and EC1-PEG

The shift in the intrinsic fluorescence emission maximum was monitored to evaluate changes in the microenvironment of the Trp6 and Trp66 residues in the EC1-IN, EC1-PEG, and EC1 proteins upon incubation at 4 °C in pH 3.0, 7.0, and 9.0 for 28 days (Table 1). The Trp emission profiles can provide an indication of the buried or exposed nature of the Trp residues as well as the tertiary structural properties of the protein. At pH 3.0, a blue shift (3.0 nm) was observed in the emission spectra of EC1 between day 0 and day 28, and this blue shift suggests the possibility burial of Trp residues in an apolar environment due to protein aggregation. In contrast, the blue shift in EC1-IN was smaller (0.7 nm) than that in EC1 at pH 3.0.

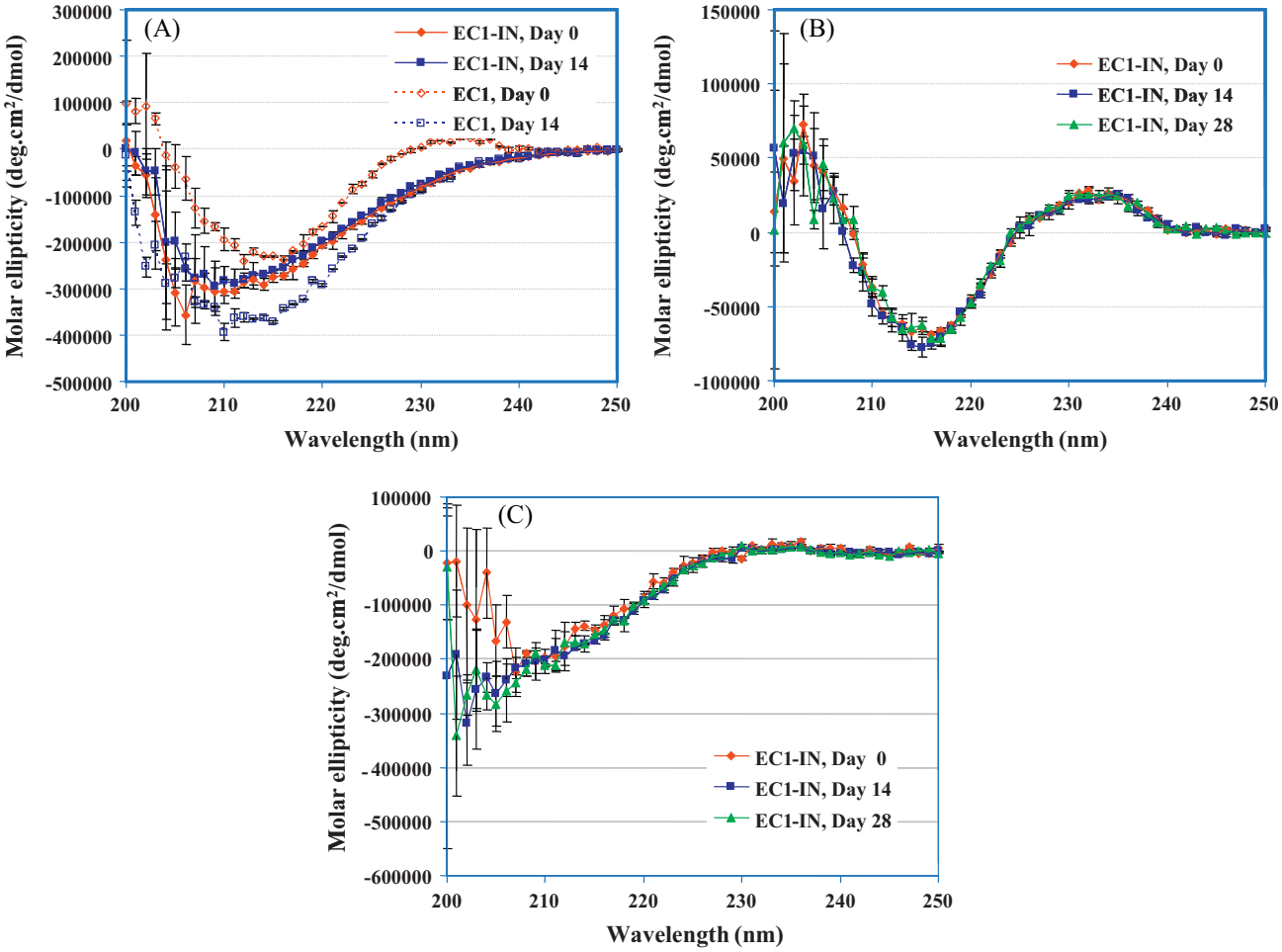

Fig. 6. (A) Comparison of the CD spectra of EC1-IN and EC1 after their incubation at pH 3.0 for 14 days at 4 °C. The CD spectrum of EC1-IN does not change substantially after 14 days, whereas the spectrum of EC1 changes upon 14 days incubation. (B) The CD spectra of EC1-IN after its incubation at pH 7.0 for 14 and 28 days at 4 °C. The CD spectrum of EC1-IN does not change substantially after 14 and 28 days. (C) The CD spectra of EC1-IN after incubation at pH 9.0 for 14 and 28 days at 4 °C. The CD spectra of EC1-IN after 14 and 28 days are substantially different from those on day 0.

At pH 7.0, a large red shift (6 nm) was observed in the EC1 spectra, and only a small blue shift (~1.0 nm) was observed in EC1-IN spectra after 28 days incubation. The red shift indicates the exposure the Trp residue(s) to the solvent upon unfolding. Only a small shift was observed for emission maxima (~1.0 nm) for both EC1 and EC-IN upon incubation for 28 days at pH 9.0. Using the maximum intensity against temperature, thermal unfolding curves of EC1-IN and EC1 were obtained upon incubation for 0 and 28 days at 4 °C and pH 7.0. EC1 had one transition around 35 °C on day 0, but displayed two transitions after incubation for 28 days with the first transition at 35 °C and the second at above 60 °C with a shift to very high wavelengths (>375 nm). Overall, the fluorescence data obtained suggest that at pH 3.0, 7.0 and 9.0, the microclimate of the Trp residues in EC1-IN does not change significantly upon incubation for 28 days at 4 °C. In contrast, emission spectra of EC1 at pH 3.0 and 7.0 and its thermal unfolding at pH 7.0 showed significant changes under the same incubation conditions.

Table 1
The wavelength at the maximum intensity fluorescence emission.

	EC1 (λ in nm)	EC1-IN (λ in nm)	EC1-PEG (λ in nm)
pH 3.0			
Day 0	339.93 ± 0.04	340.61 ± 0.08	339.87 ± 0.03
Day 28	337.09 ± 0.04	338.91 ± 0.06	
pH 7.0			
Day 0	339.27 ± 0.03	342.13 ± 0.05	339.83 ± 0.05
Day 28	345.81 ± 0.13	341.21 ± 0.05	
pH 9.0			
Day 0	342.0 ± 0.0	343.0 ± 1.0	339.97 ± 0.02
Day 28	339.67 ± 1.15	341.67 ± 0.58	

Intrinsic fluorescence emission spectra of EC1-PEG between 305 nm and 405 nm after excitation at 295 nm were compared to the EC1 spectra at pH 3.0, 7.0, and 9.0 (Table 1). The wavelength of maximum emission at pH 3.0 was 339.87 ± 0.03 nm for EC1-PEG, similar to that for EC1 (339.93 ± 0.04 nm) at pH 3.0. At pH 7.0, it was 339.83 ± 0.05 nm, similar to that of EC1 (339.27 ± 0.03 nm) at pH 7.0. At pH 9.0, however, there was a small but significant difference in the wavelengths of maximum emission of EC1-PEG (339.97 ± 0.02 nm) and EC1 (341.31 ± 0.03 nm). A possible explanation for this difference at pH 9.0 is that the PEG interacts with at least one of the two Trp residues, probably the Trp6 residue that is near the site of PEGylation at the Cys13 residue. The presence of the bulky PEG group near the Cys13 residue makes Trp6 less exposed to the solvent in EC1-PEG than in EC1.

4. Discussion

Our long-term goal is to understand the in vitro and in vivo biological functions of the EC1 domain in inhibiting homotypic and heterotypic cell–cell adhesion. Because of the instability of EC1, it is necessary to find a method for stabilizing it for its long-term use in

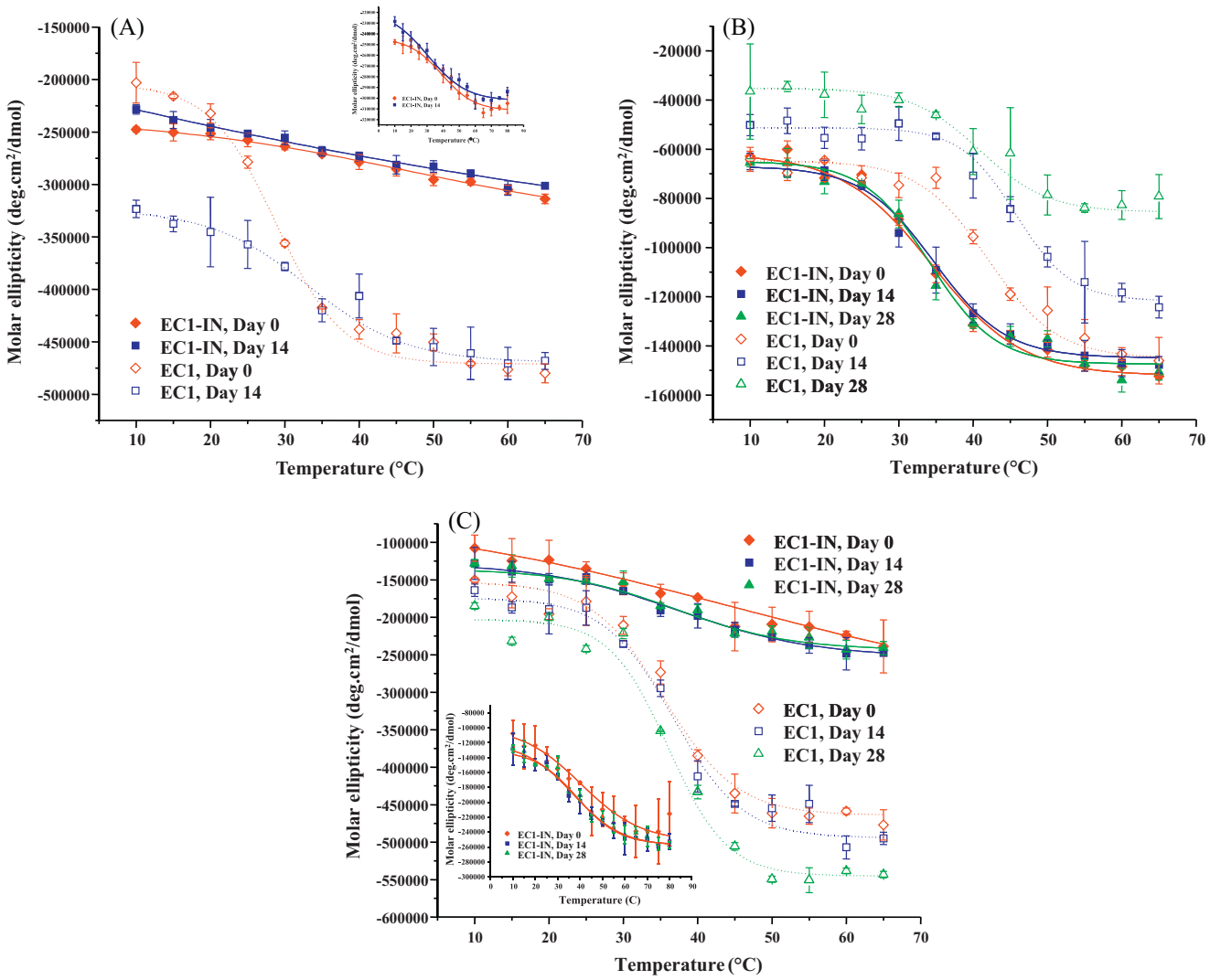

Fig. 7. Comparison of the thermal unfolding curves of EC1-IN and EC1 proteins after incubation for 14 days at (A) pH 3.0, (B) pH 7.0, and (C) pH 9.0. There is a very small difference in the thermal unfolding curves of EC1-IN, whereas a clear change is seen in the thermal unfolding curve of EC1. The inset shows that the thermal unfolding curves of EC1-IN can be fitted to sigmoidal functions. Thermal unfolding profile of EC1 and EC1-IN measured by the CD signal at 218 nm after incubation for 0, 14, and 28 days at 4 °C at pH 7.0. There are limited differences in the thermal unfolding curves of EC1-IN, but dramatic differences are seen in the thermal unfolding curves of EC1. EC1 has a higher transition temperature than EC1-IN, indicating that EC1-IN is less stable to thermal unfolding than EC1. Thermal unfolding profile of EC1 and EC1-IN measured by the CD signal at 218 nm after incubation for 0, 14, and 28 days at 4 °C and pH 9.0. The thermal unfolding curves of EC1-IN are substantially different from those of EC1. The inset shows that the thermal unfolding of EC1-IN can be fitted to sigmoidal functions.

biological assays. One way to prevent the aggregation/precipitation of EC1 is by inhibiting the covalent dimerization of EC1 via the Cys13 residue (Makagiansar et al., 2002b). The X-ray structure of EC1 shows that the Cys13 residue is exposed on the surface of EC1 (Nagar et al., 1996); it is adjacent to Trp6, which can cause domain swapping between two EC1 molecules to form a physical dimer (Pokutta et al., 2008). Molecular modeling studies indicate that the formation of a covalent dimer of EC1 (EC1-ss-EC1) is a facile reaction (Makagiansar et al., 2002b). We have shown that the formation of EC1-ss-EC1 dimer increases the dynamic properties of the EC1 unit compared to the EC1 monomer (Trivedi et al., 2009). The dynamic nature of EC1-ss-EC1 may contribute to its susceptibility to peptide bond hydrolysis and aggregation reactions. Thus, alkylation of the Cys13 residue could prevent intermolecular disulfide bond formation that leads to aggregation of EC1.

Here, we have demonstrated that the thiol group of Cys13 can be modified to a thioether by alkylation reaction with iodoacetate, iodoacetamide, and maleimide-PEG to generate EC1-IA, EC-IN, and EC1-PEG, respectively. The PEG group was conjugated via nucleophilic attack of the maleimide group by the thiol of the Cys13 residue on EC1 to produce EC1-PEG. This reaction produced the PEGylated product with a single site of modification as determined by HPLC and mass spectrometry. Although the Cys13 residue is exposed on the surface of EC1, the alkylation reaction of the thiol group of the folded form of EC1 was slow; the reaction was accelerated by partially unfolding the protein by incubation in 4.0 M Gdn.HCl solution. Gradient removal of Gdn.HCl refolded the EC1-IN and EC1-PEG properly to provide a structure similar to that of the parent EC1 protein as determined by CD. Unfortunately, while EC1-IA was successfully synthesized, it did not refold properly. Therefore, EC1-IA was not investigated further for stability studies. The additional negative charge on the acetate group of EC1-IA may prevent its proper refolding due to destabilization of the refolding intermediate via repulsion of negative charges or stabilizing the undesirable intermediate via charge–charge interactions (i.e., salt bridge). A similar result has been observed in the derivatization of sorghum phosphoenolpyruvate carboxylase (PEPC) mutant

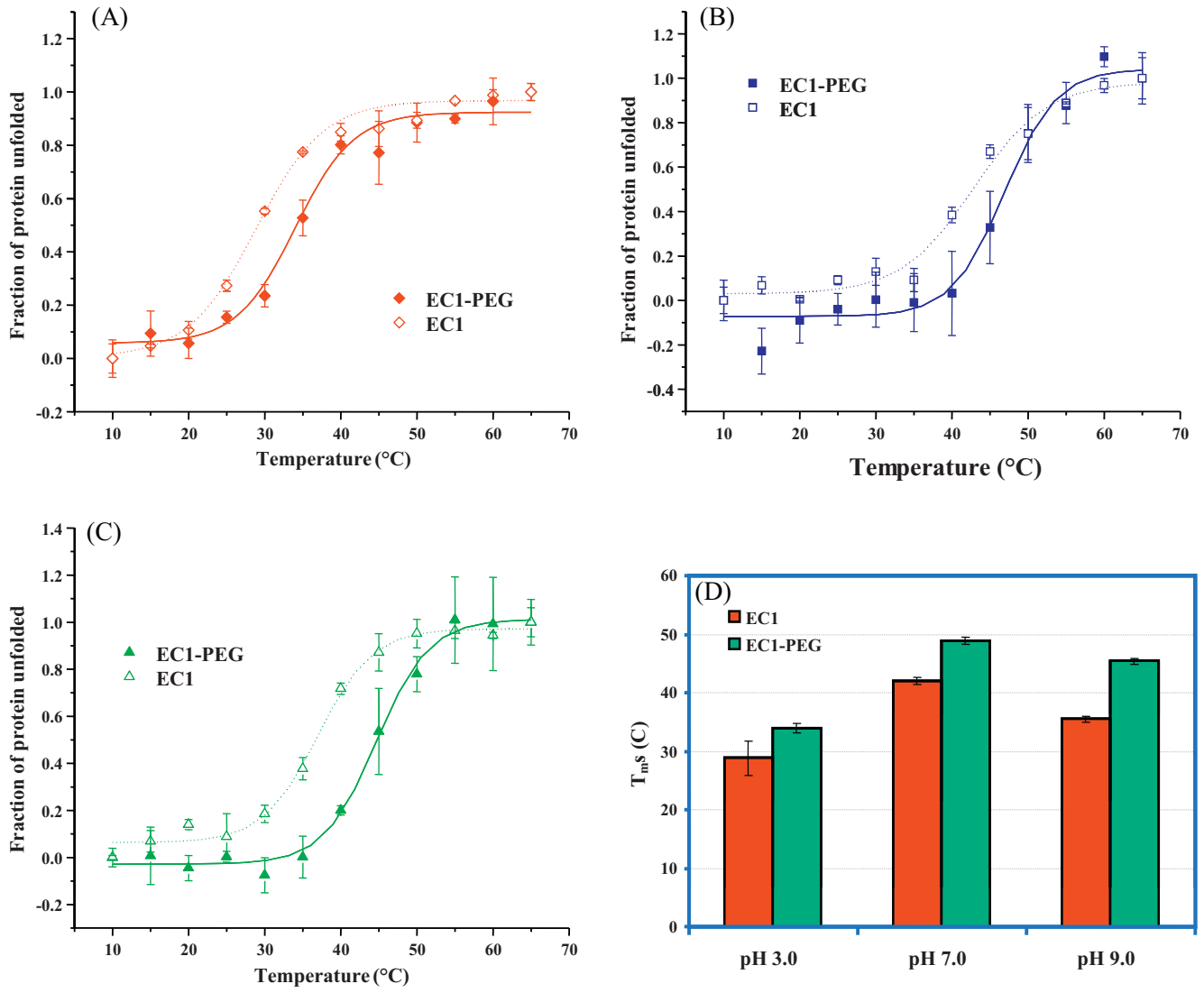

Fig. 8. Thermal unfolding profile of EC1-PEG compared to that of EC1 obtained by plotting the CD signal at 218 nm against the corresponding temperature at (A) pH 3.0, (B) pH 7.0, and (C) pH 9.0. (D) A comparison of the unfolding temperatures (T_ms) for EC1-PEG and EC1 at pH 3.0, 7.0, and 9.0 after fitting the CD thermal unfolding profiles to sigmoidal functions.

with iodoacetate; the derivatized product has decreased enzymatic activity (Duff et al., 1993). In contrast, its derivatization with iodoacetamide did not change the enzymatic activity of the enzyme (Duff et al., 1993). This result suggests that this difference in enzymatic activity can be attributed to the introduction of a negative charge in PEPC upon iodoacetate derivatization but no additional negative charge when derivatized with iodoacetamide.

Although EC-IN has a CD spectrum similar to that of EC1 at pH 7.0, the CD spectra of EC1-IN are sensitive to pH changes. At pH 7.0, the EC1-IN has estimated secondary structure content similar to that of EC1 from the CD spectra; EC1-IN has 47% β-sheet, 5% α-helix, and 48% random coil and EC1 has 51% β-sheet, 2% α-helix, and 47% random coil. On the other hand, the CD spectra of EC1-IN were dramatically different from those of the EC1 protein at pH 3.0 and 9.0. The sensitivity of EC1-IN to pH changes could be due to the different electronic properties and hydrogen bonding potential of the amide group of derivatized Cys13 on EC1-IN compared to the parent thiol group of Cys13 on the EC1 protein. The thiol group of Cys13 in EC1 can be ionized to thiolate ion (pKa around 9.1) and it can form electrostatic interactions with surrounding functional groups. In contrast, the amide group of the derivatized Cys13 cannot be ionized and may not form efficient interactions with surrounding functional groups. As noted previously, the thiol group of Cys13 was not very reactive in the folded form of EC1 and this thiol group could only be derivatized in the unfolded form. This observation suggests that the thiol group of Cys13 is involved in hydrogen bonding and/or electrostatic interactions with surrounding functional groups to stabilize EC1 tertiary structure. Altered secondary structures of proteins upon derivatization have been observed previously in thiol-alkylated ovalbumin using iodoacetate and iodoacetamide. The alkylated ovalbumin and BSA have a decreased α-helical content and an increased β-sheet content (Batra et al., 1989a,b).

The stability of EC1-IN and EC1 monitored by CD and fluorescence indicates that EC1-IN is more stable than EC1 under pH 3.0 and 4 °C for 14 days. The fluorescence emission spectra of EC1-IN did not change upon incubation for 28 days at 4 °C, but there was a blue shift in the emission spectra of EC1 upon treatment under the same condition. The melting temperatures (T_m) of EC1-IN estimated from CD spectra at both pH 3.0 and 9.0 are higher than the T_m of EC1. For example, the T_m of EC1-IN is 39.9 ± 1.0 °C, whereas the T_m of EC1 is 28.8 ± 0.3 °C at pH 3.0. At pH 7.0, however, the T_m of EC1-IN (33.9 ± 0.7 °C) is lower than the T_m of EC1 (42.0 ± 0.6 °C). A decrease in thermal stability has been reported previously upon

Cys residue alkylation of alpha-alpha tropomyosin (AAT) (Holtzer et al., 1990). It is interesting to find that although EC1-IN has a lower T_m than EC1 at pH 7.0, incubation of EC1-IN at 4 °C for 28 days did not result in any change in CD spectra. This is in contrast to incubation of EC1 under the same conditions, which produced different CD spectra due to aggregation (Trivedi et al., 2009). These results also support the idea that the presence of the free thiol group in EC1 catalyzed its instability by forming a covalent dimer, and the prevention of covalent dimerization inhibited the formation of protein aggregates and precipitates. Therefore, the T_m alone may not be sufficient for determining the instability of a protein.

Because EC1-IN has different conformations than the parent EC1 at pH 3.0 and 9.0 and also has a lower T_m than the parent EC1 at pH 7.0, an alternative alkylation method such as PEGylation was utilized to improve the stability of EC1 (Morar et al., 2006). In this case, the thiol group of the Cys13 residue was reacted with maleimide polyethylene glycol (PEG) to make EC1-PEG, and its structural characteristics were evaluated using spectroscopic methods. PEGylation of the Cys residues of proteins has been employed previously to improve protein in vitro and in vivo stabilities (Bell et al., 2008; Colonna et al., 2008; Long et al., 2006; Rosendahl et al., 2005a,b; Tsutsumi et al., 2000; Vanwetswinkel et al., 2000). PEGylation also does not change the protein secondary structure compared to that of the parent protein (Digilio et al., 2003; Hinds and Kim, 2002). Far UV CD studies showed some minor differences between CD spectra of EC1-PEG and EC1 at pH 3.0. At pH 7.0 and 9.0, however, no substantial differences were observed between the CD spectra of EC1-PEG and EC1. Compared to EC1-IN, the secondary structure of EC1-PEG is less sensitive to pH changes. The thermal unfolding profiles determined by CD spectroscopy suggest that EC1-PEG is significantly more stable than EC1; the increases in T_m of EC1-PEG compared to EC1 are 5.1 °C at pH 3.0, 4.0 °C at pH 7.0, and 7.7 °C at pH 9.0 (Fig. 8). A similar trend was observed by plotting the change in fluorescence wavelength maximum as a function of temperature. The EC1-PEG protein was more stable than EC1-IN or EC1 after incubation for 4 h at 70 °C at all three different pH values (3.0, 7.0 and 9.0). EC1-IN was more stable than EC1 at pH 7.0 and 9.0, but both proteins were equally unstable at pH 3.0. As with EC1 at pH 3.0 and 70 °C, EC1-IN was chemically degraded via peptide bond hydrolysis at Asp93–Pro94, and only 22% of EC1-IN remained in solution after 4-h incubation. Only 10% of the hydrolysis product was observed in solution because the degradation involved precipitation of the protein. All the methods examined so far indicate that PEGylation is the best strategy for stabilizing the EC1 protein. It is also possible that the PEGylation of EC1 decreases the dynamic properties of EC1-PEG, which contributes to the decrease in the hydrolysis reaction; however, this proposal needs further investigation.

PEGylation of therapeutic proteins has been shown to improve their physical and biological properties. PEGylated proteins have a larger hydrodynamic radius with lower glomerular filtration, lower immunogenicity, and a higher enzymatic stability compared to the parent protein. It was proposed that PEGylation at several sites on a protein with higher molecular weight branched PEGs as opposed to linear PEG can form a polymer shell around the protein; this recruits water to form an outer water shell that minimizes intermolecular protein interactions (Long et al., 2006; Morar et al., 2006; Rosendahl et al., 2005a,b; Tsutsumi et al., 2000; Vanwetswinkel et al., 2000). Thus, PEGylation of therapeutic proteins produces a higher protein systemic circulation, and the amount and frequency of dosing can be reduced. In EC1-PEG, the PEG group may not be large enough to form a shell around the entire EC1 molecule because it is a linear 5000 Da PEG. The X-ray structure of EC1 shows that the Trp6 and Cys13 residues are located at the dimerization surface. The X-ray structure of EC1 of C-cadherin shows that the equivalent Trp residue is involved in domain swapping between two EC1 domains. In other words, the Trp residue on one EC1 is buried in a hydrophobic pocket of another EC1 to form a physical dimer (Boggon et al., 2002). It has been suggested that EC1 could have two homophilic binding surfaces, one at the domain-swapping region at the N-terminal and the other in the region where the His–Ala–Val (HAV) and Ala–Asp–Thr (ADT) sequences are located (Makagiansar et al., 2001; Sinaga et al., 2002). Thus, we propose that the presence of the PEG on EC1-PEG near the domain-swapping region may prevent the formation of physical and chemical dimers of EC1 of E-cadherin. However, the presence of PEG group Cys-13 may not interfere with HAV region of EC1-PEG for binding to E-cadherin molecules on the cell surface to block cell–cell adhesion. In addition, EC1-PEG can be used to elucidate the role of the surface opposite to the PEGylation surface (HAV and ADT surface) on E-cadherin in mediating homophilic and heterophilic cell–cell adhesion (Kiptoo et al., 2011; Kobayashi et al., 2006; Makagiansar et al., 2001; Sinaga et al., 2002). It is not yet clear whether the size of PEG molecules can influence the stability and bioactivity of the EC1-PEG protein. However, the increase in size of PEG molecules can increase the vivo systemic circulation of PEGylated protein; this is presumably due to the increase of the hydrodynamic radius of the PEGylated protein.

In conclusion, alkylation of the Cys residue in EC1 improves the stability of the EC1 domain, indicating that the formation of a covalent dimer of EC1 is crucial for the instability of EC1. The EC1-PEG is the best modification for improving chemical and physical stability and long-term storage of EC1. Unlike the modified EC1-IN protein, EC1-PEG has a structure similar to that of the parent EC1 at different pH values. In the future, the biological activity of EC1-PEG will be compared to that of EC1-IN and EC1 in inhibiting E-cadherin-mediated homotypic and heterotypic cell–cell adhesion in in vitro cell culture systems developed in our laboratory (Kobayashi et al., 2006).

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