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Liquid chromatographic and capillary electrophoretic examination of intact and degraded fusion protein CTLA4Ig and kinetics of conformational transition

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Abstract

Methods have been developed for the CE and HPLC analysis of CTLA4Ig, an immunoglobulin fusion protein. Two different LC approaches, size-exclusion (SEC) and "mixed-mode" ion-exchange (ABx), were developed along with a CE method that uses a micellar electrokinetic chromatographic buffer consisting of borate ions, sodium dodecyl sulfate and acetonitrile. These assays measure the presence of several CTLA4Ig-related species, and the observed changes resulting from multiple modes of degradation. In an attempt to identify possible degradation products, collections were taken from the ABx and SEC liquid chromatographic systems and further analyzed by matrix-assisted laser desorption time-of-flight (MALDI TOF) mass spectrometry. Multiple species were detected covering a wide molecular mass range. In addition, the CE method was used to study conformational kinetics between two forms of CTLA4Ig and to estimate the activation energy of the conformer–conformer transition. Pseudo-first-order reaction kinetics were demonstrated for CTLA4Ig samples stressed with papain, H_2O_2, and sodium dodecyl sulfate/heat.

Keywords: Capillary electrophoresis; Conformer–conformer transition; Fusion protein CTLA4Ig; Immunoglobulin fusion protein; Proteins

1. Introduction

Recombinant DNA techniques that produce highly complex biomolecules require analytical procedures capable of detecting minor variations in peptides and proteins (typically present in dilute solutions). HPLC and CE, utilized individually or in combination, provide information for determining drug-related impurities, main peak assays, or chiral analyses. In addition, these methods may be used not only to assess the initial purity of a proteinaceous species, but to conduct time-dependent studies. Kinetics of the degradation of pharmaceutical proteins may be performed with stability-indicating CE and HPLC analytical methods [1,2].

CTLA4Ig is an immunoglobulin fusion protein, made by joining the extracellular domain of

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the human CTLA4 receptor with the first two constant chains (the variable-like domain in IgG, specifically, the hinge CH2 and CH3 domains) of the human IgG 1 chain [3]. CTLA4 is a membrane receptor on cytotoxic T cells. A counter receptor, B7, is located on antigen presenting cells and, together with CTLA4, forms a co-stimulatory pathway for immune response. CTLA4Ig plays a role in T cell responses in vitro and in vivo by specifically binding to the B7 ligand on antigen presenting cells and inhibiting T cell responses. This fusion protein is a potent inhibitor of in vitro immune functions requiring T and B cell cooperation. The interaction between B7 and CTLA4Ig is an important co-stimulant needed to elicit a full immune response, including antibody production [4]. By the nature of its immune function and inhibitory behavior, CTLA4Ig thus has the potential to be used clinically as an immunosuppressant. Analytical methods were required to analyze CTLA4Ig produced from cell cultures.

Fig. 1 displays the general structure of the protein. CTLA4Ig is a homo-dimer containing 356 amino acids [5] with a p*I* range of ca. 5.1–6.6, and M_r ca. 92 000. The theoretical primary structural mass is 78 000 with the remainder of the mass contained in carbohydrate attachments. Through the recombinant production process, various forms of CTLA4Ig can be produced, for example: glycoforms, conformational isomers, and primary sequence variants. Analysis of the fusion protein requires, therefore, that a variety of species be examined to ascertain purity and stability. Different analytical methods can be combined to evaluate these factors of the drug substance itself. Theoretically, orthogonal procedures such as capillary electrophoresis (CE) and liquid chromatography (HPLC) provide a complementary, and, hence, more complete characterization of stability and purity.

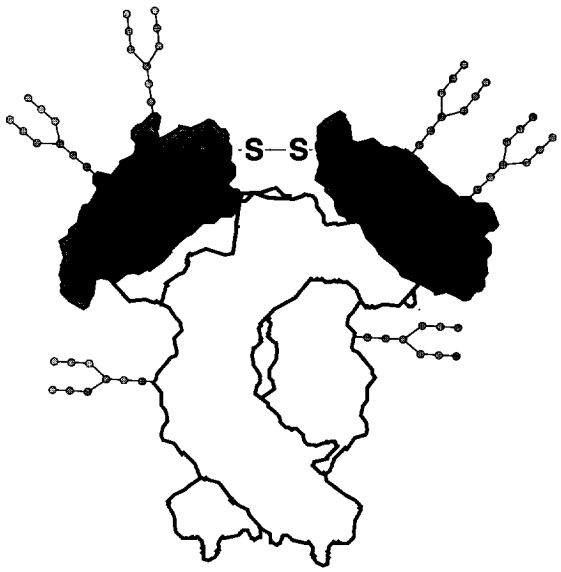

Fig. 1. Representative structure of CTLA4Ig. The gray region is the CTLA4Ig portion (B7 binding region), the white region is the human Ig portion; the branched structures represent the carbohydrate portions of the molecule.

In this paper, protocols have been developed for the CE and HPLC analysis of CTLA4Ig. Two different LC methods are employed, namely, size exclusion and "mixed-mode" ion exchange. The CE method uses a micellar electrokinetic chromatography (MEKC) buffer consisting of borate ions, sodium dodecyl sulfate (SDS) and acetonitrile. These assays measure the presence of several CTLA4Ig-related species, and the observed changes resulting from multiple modes of degradation. Many primary structure variations are possible for a large protein, e.g., deamidation of Asn or Gln residues, carbohydrate variation and alteration, and fragmentation of peptide chains. In addition, the MEKC method was used to study conformational kinetics between two forms of CTLA4Ig and to estimate the activation energy of the transition.

2. Experimental

2.1. Reagents and materials

Certified ACS sodium borate crystals, sodium acetate, methanol, acetonitrile, 30% hydrogen peroxide, glacial acetic acid, phosphoric acid and 10 *M* sodium hydroxide were obtained from Fisher Chemical (Fair Lawn, NJ, USA). Ultra pure MES [2-(N-morpholino)ethanesulfonic acid], sodium azide, sodium dodecyl sulfate (SDS), bovine albumin dimer, trypsin, papain

and α-chymotrypsin were purchased from Sigma (St. Louis, MO, USA). Sodium sulfate was obtained from Mallinckroft (Paris, KY, USA), and potassium phosphate, monobasic from J.T. Baker (Phillipsburg, PA, USA). Sinapinic acid was obtained from Aldrich (Milwaukee, WI, USA). In the preparation of all buffer and sample solutions, 18 MΩ water from a Milli-Q filtration system (Waters, Milford, MA, USA) was used.

2.2. CE instrumentation and methods

A Beckman P/ACE 2100 CE system (Palo Alto, CA, USA) controlled by an IBM PS/2 with either P/ACE software and a Microsoft Windows interface or Beckman System Gold software was used throughout this study. Multichrom (VG Data Systems, Fisons Instruments, Cheshire, UK) was also used to determine peak heights and areas. Pretreated fused-silica capillaries (50 or 75 μm I.D., 50 cm effective length measured to the optical window, with a total length of 57 cm, Beckman Instruments (Palo Alto, CA, USA)) were used for all MEKC experiments. The electropherograms were monitored at 214 nm with a data collection rate of 10 Hz. The CE buffer consisted of 14 m*M* sodium borate, 17 m*M* SDS, and 3.5% acetonitrile (pH 9.4). Similar CE buffers have previously been used for the analysis of very-high-molecular-mass (antibody-related) proteins [6]. The capillary cartridge temperature was maintained at 25°C. Samples were injected in the low-pressure mode for 5 s. Electropherograms were redrawn using Microsoft Excel, and in some cases reanalyzed using Multichrom.

2.3. HPLC instrumentation and methods

The HPLC system consisted of a Perkin-Elmer advanced LC sample processor ISS 200 and a binary LC pump (Norwalk, CT, USA) with a Waters 991 photodiode array detector. SEC experiments were performed using a Tosohaas TSK gel column (30 cm × 7.8 mm I.D.) in conjunction with a prepacked TSK-gel guard column SW (4.0 cm × 6.0 mm I.D.) (Tosohaas, Montgomeryville, PA, USA). The mobile phase for the SEC determinations consisted of an aqueous solution of 0.1 *M* sodium sulfate, 0.1 *M* potassium phosphate, monobasic, and 0.05% sodium azide (pH 6.7) at a flow-rate of 1 ml/min.

A Bakerbond ABx "mixed-mode" ion-exchange column, (50 × 4.6 mm, 5 μm, 300 Å) (J.T. Baker), containing weak cation-exchange groups, a low concentration of anion-exchange groups, and hydrophobic ligands, was also used to evaluate CTLA4Ig samples. Mobile phase A consisted of 0.025 *M* MES (pH 5.5) and mobile phase B contained 0.65 *M* sodium acetate, 20% methanol (pH 7.0). The gradient varied from 0% B to 20% B in 40 min at a flow-rate of 1 ml/min.

2.4. MS instrumentation and methods

Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectra were acquired on a Bruker Reflex mass spectrometer equipped with a 337 nm nitrogen laser (Bruker Analytical Systems, Billerica, MA, USA). The spectra were acquired in the linear mode and represent the sum of 20 laser shots. All ions were desorbed at a laser power just above the threshold value needed to produce ions and were accelerated by an extraction voltage of 30 kV. The matrix was a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in water–acetonitrile (2:1, v/v). Samples were diluted 5:1 or 10:1, depending on concentration, with the matrix solution, and 0.5 μl of the mixture was then deposited onto the sample stage and allowed to air dry. Low-mass matrix ions were deflected by the application of a voltage pulse at 5 μs. Mass assignments were established on peaks centroided at 80% of maximum height. Mass calibration was performed externally against the singly- and doubly-charged ions of bovine albumin dimer. While it is difficult to assess the absolute mass accuracy, due to uncertainty in the true molecular mass of CTLA4Ig, values of ≤0.1% were routine for well characterized, homogeneous proteins. It must also be considered that, due to the limited resolution of MALDI-TOF ($m/\Delta m \approx 20$ for $[M + H]^+$ of CTLA4Ig, at full width/half maximum), the experimentally determined M_r was actually the average for a

mixture of various heterogeneous forms. The standard deviation in a typical measurement of CTLA4Ig was ±0.2% (200 a.m.u.).

2.5. CD measurements

The circular dichroism (CD) spectra were measured with a Jasco spectropolarimeter, Model J-600 (Jasco, Easton, MD, USA). A jacketed cylindrical cuvette of 0.5 mm light-path length was used for the near and far-UV range measurements. Temperature regulation was carried out by water flow through the cell using a NESLAB circulator and thermoregulator Model RTE-110 (Neslab Instruments, Newington, NH, USA). After each temperature adjustment and stabilization (within ±1°C), approximately 5 min were allowed for thermal equilibration between the circulating water and the protein solution in the cell before the first scan of the spectrum was collected. The CTLA4Ig concentration in 12 m*M* borate buffer, pH 9.4 was 1.0 mg/ml. In all experiments where SDS was utilized, the concentration was 1 mg/ml. A total of 32 scans were averaged for both sample and buffer blanks. After the buffer spectrum had been subtracted, the results were converted into molar ellipticity using a molecular mass of the CTLA4Ig of 92 000 and a total number of amino acids of 712. Protein concentrations were measured spectrophotometrically using an absorptivity of 1.1 at 280 nm.

2.6. Selected methods for sample degradation

Degradation of CTLA4Ig samples was accomplished using five different methods. The first method used H_2O_2 as an oxidizing agent (5 μl of 30% H_2O_2, 50 μl sample, and 50 μl water). The second degradation method involved heating the sample at two different temperatures: 70°C for 20 min and 50–60°C for 10 min. In the third method, the pH of the CTLA4Ig sample was adjusted with either acetic acid (to pH 4) or NaOH (to pH 10) for acid and base-catalyzed hydrolysis, the original pH of the sample being 7.5 in a 25 m*M* phosphate/50 m*M* NaCl buffer. The fourth degradation method used partial enzymatic digestion with papain, trypsin, or α-chymotrypsin. Specifically, 250 μl Tris buffer (pH 8), 230 μl water, 80 μl sample, 50 μl α-chymotrypsin (1 mg/ml); 250 μl Tris buffer (pH 8), 230 μl water, 83 μl sample, 1 μl typsin (0.65 mg/ml); and 250 μl Tris buffer (pH 8), 230 μl water, 41 μl sample, and 20 μl papain (1 mg/ml) were used for partial digestion. The last protocol required dissolution of CTLA4Ig sample in 14 m*M* sodium borate, 7 m*M* SDS (pH 9.4), followed by heating the sample to 30, 40, 50, 61, or 71°C.

3. Results and discussion

3.1. HPLC method development

A number of ion-exchange stationary phase chemistries were examined. It is interesting to note that the mixed-mode anion exchanger, ABx, yielded the most useful separation. A variety of gradients were examined using the ABx column to optimize the separation, with the final conditions being 0% to 20% mobile phase B in 40 min, see Fig. 2A. Mobile phase A consisted of 0.025 *M* MES (pH 5.5) and mobile phase B contained 0.65 *M* sodium acetate, 20% methanol (pH 7.0). One major peak with several unresolved peaks was obtained with the ABx system within 30 min.

The SEC separations were accomplished using the procedure recommended by the manufacturer with no further optimization. The SEC system produced a minor and a major peak in 13 min, with the minor peak assumed to be an aggregated (perhaps dimeric) species, Fig. 2B. Standard acetonitrile–trifluoroacetic acid gradient reversed-phase LC conditions for protein analysis yielded a low resolution peak for CTLA4Ig and this procedure was not pursued further.

3.2. CE method development

For the CE separations, a variety of detergents were evaluated in an attempt to examine native CTLA4Ig forms and isoform impurities (glycoforms, conformational isomers, or primary se-

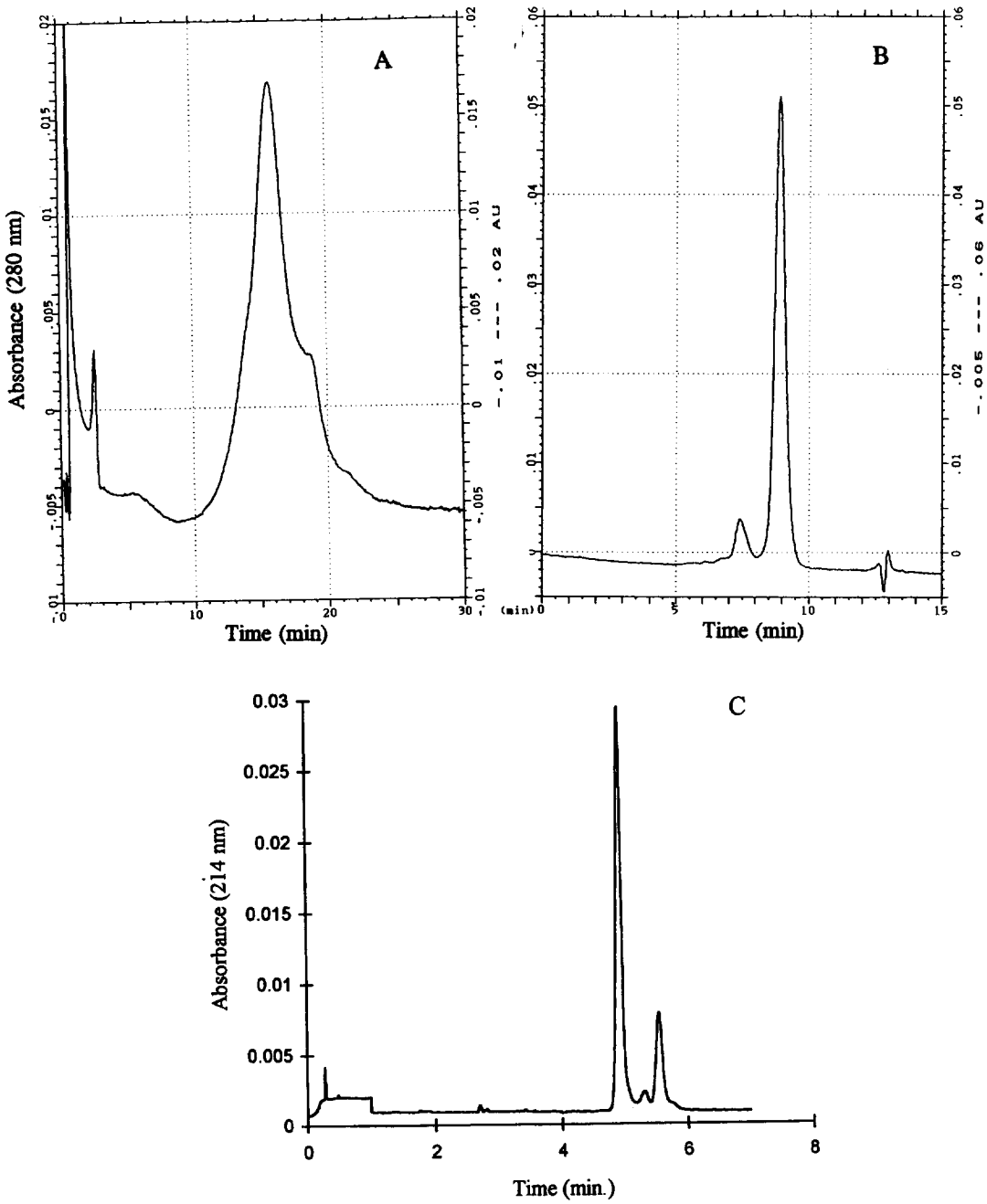

Fig. 2. Separation of intact CTLA4Ig by three methods: (A) ABx, (B) SEC, and (C) MEKC. Conditions for (A): ABx "mixed-mode" ion-exchange column, 50 × 4.6 mm, 5 μm, 300 Å; mobile phase A = 0.025 *M* MES (pH 5.5) and mobile phase B = 0.65 *M* sodium acetate, 20% methanol (pH 7.0); the gradient varied from 0% B to 20% B in 40 min at a flow-rate of 1 ml/min. Conditions for (B): Tosohaas TSK gel column, 30 cm × 7.8 mm, in conjunction with a prepacked TSK-gel guard column SW, 4.0 cm × 6.0 mm I.D.; mobile phase consisted of 0.1 *M* sodium sulfate, 0.1 *M* potassium phosphate, monobasic, and 0.05% sodium azide (pH 6.7) at a flow-rate of 1 ml/min. Conditions for (C): fused-silica capillary, 75 μm I.D., 50 cm effective length measured to the optical window, with a total length of 57 cm; 14 m*M* sodium borate, 17 m*M* SDS, and 3.5% acetonitrile (pH 9.4), 214 nm, 25°C.

quence variants). A buffer containing borate ions, SDS, and acetonitrile produced the best resolution of species, Fig. 2C. This CTLA4Ig profile exhibited two major and one minor peak attainable within an 8-min analysis time. The concentration of SDS was varied above and below the critical micelle concentration (CMC) of 8 m*M*, Table 1. The amount of detergent present in the buffer played a role in the selectivity of the procedure. At SDS concentrations below the CMC, decreased selectivity was observed but not lost, with the first peak dominating the electropherogram. Increases in the SDS concentration above 17 m*M* did not significantly improve the separation (see Table 1), but did increase the operating current substantially and distort the peak profiles. The area reproducibility for multiple injections of CTLA4Ig gave an average percent R.S.D. of 0.88% [the different samples yielded R.S.D. percents of 0.75% ($n = 4$), 0.96% ($n = 3$), 0.68% ($n = 3$), 1.12% ($n = 3$), 0.90% ($n = 3$)], values consistent with general CE analysis.

As part of any analytical validation procedure, the method must show specificity. The use of impurity-generating processes, such as heat, chemical stress (i.e. changing pH), oxidation, reduction, and enzymatic cleavage, test the specificity of the method to chromatographically or electrophoretically separate degradation products. CTLA4Ig samples were stressed either chemically or enzymatically to test the selectivity of the ABx, SEC, and MEKC methods. Representative chromatograms showing the degradation products by the ABx and MEKC methods are shown in Figs. 3 and 4. The ABx and the MEKC separations yielded mutually complementary information, with the MEKC system providing higher resolution in a shorter analysis time. The SEC separations, on the other hand, gave minimal information for unstressed samples, but were useful to monitor major changes in CTLA4Ig degradation.

Table 1
Selectivity values as a function of SDS concentration

SDS Concentration (m*M*)	Selectivity[a]
0	1
3.81	1.04
7.63	1.07
11.44	1.08
15.26	1.11
17.34	1.10
21.15	1.11
24.97	1.12
29.82	1.13
33.98	1.12

[a] Selectivity is defined here as the migration time ratio between the first and the second major peaks.

In an attempt to identify possible degradation products, collections were taken from the ABx and SEC liquid chromatographic systems. The collected fractions were then desalted, concentrated, and analyzed by MALDI–MS. Table 2 summarizes the major stressed and partially digested species detected by MS, indicating the presence of species covering a wide molecular mass range. The proteolytically treated samples (papain and trypsin) typically contained lower molecular mass fragments corresponding to the expected enzymatic cleavages. The ABx system was also able to resolve some lower molecular mass species, for example, from the pH 4 treated sample. The smaller peak (retention time 7.5 min) observed in the SEC separations (Fig. 2B) was attributed to an aggregated species, but upon collection and MS analysis, only intact CTLA4Ig was present, suggesting a lack of noncovalent association between the monomeric species. The other degraded samples (heat, H_2O_2 and pH 4) contained mostly intact CTLA4Ig by MS even though a shift in the major peak in the chromatograms and the electropherograms was observed (compare Figs. 2A and 3C, and Figs. 2C and 4C). This observation suggested that significant changes in hydrophobicity and net-charge can occur within the CTLA4Ig molecule without a significant (measurable) change in molecular mass.

The two major species in the CE electropherogram were determined to be of similar molecular mass. The MS data may be explained by postulating either conformational changes or slight chemical modifications in the protein (e.g., oxidation) which could produce a different net-

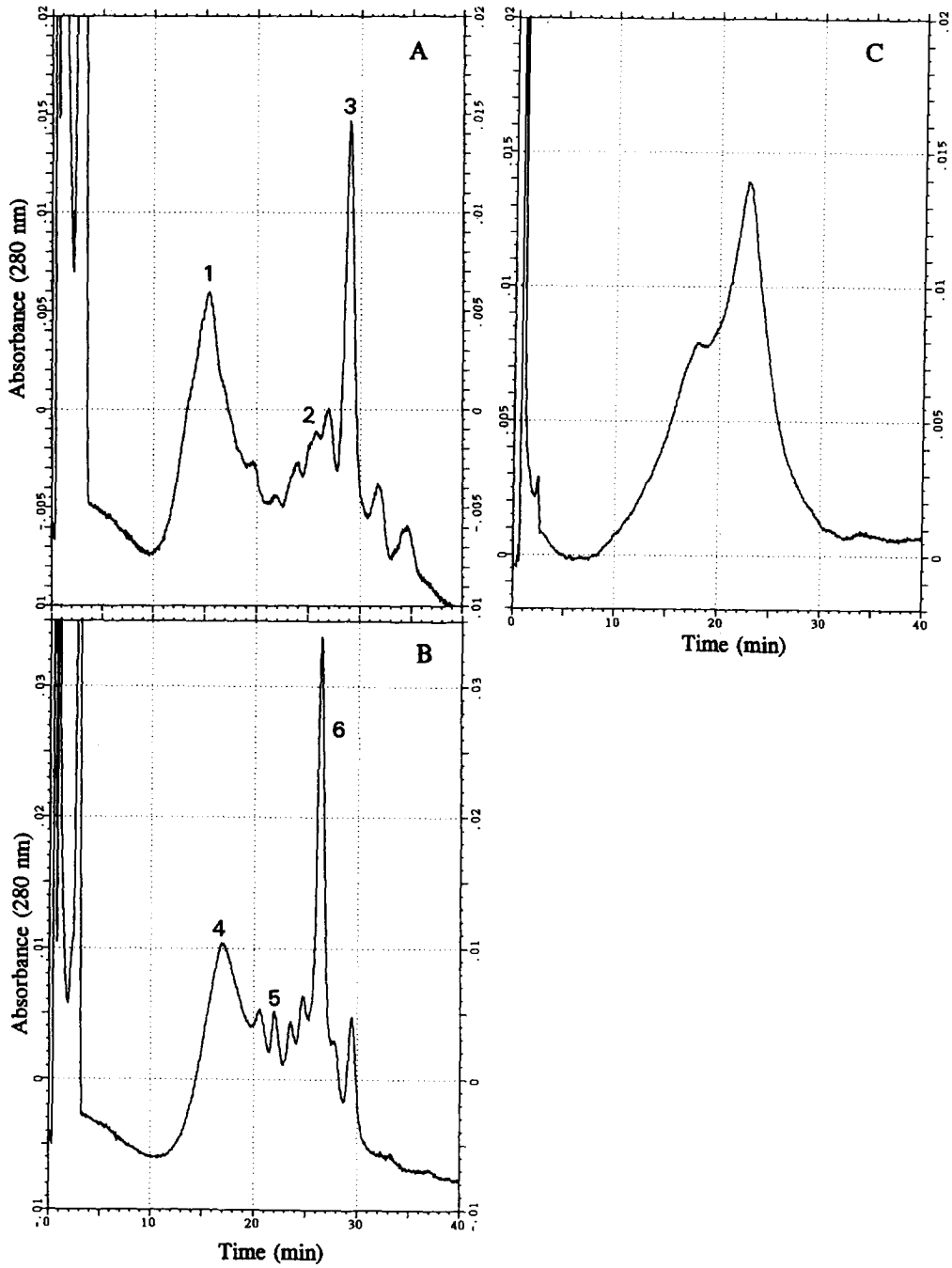

Fig. 3. Degradation of CTLA4Ig by various methods as monitored by the liquid chromatographic ABx separation: (A) trypsin digestion for 10 min; (B) papain digestion for 60 min; (C) H_2O_2 for 60 min. M_r of identified peaks are displayed in Table 3. ABx "mixed-mode" ion-exchange column, 50 × 4.6 mm, 5 μm, 300 Å; mobile phase A = 0.025 *M* MES (pH 5.5) and mobile phase B = 0.65 *M* sodium acetate, 20% methanol (pH 7.0); the gradient varied from 0% B to 20% B in 40 min at a flow-rate of 1 ml/min.

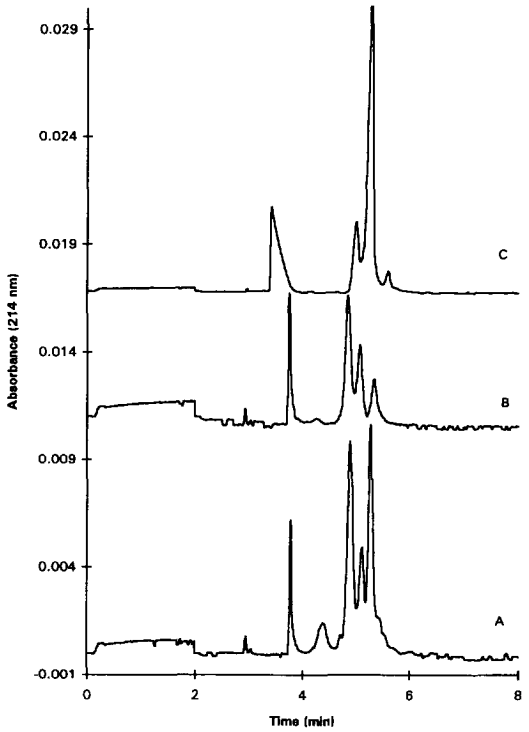

Fig. 4. Degradation of CTLA4Ig by various methods as monitored by MEKC: (A) trypsin digestion for 10 min; (B) papain digestion for 60 min; (C) H_2O_2 for 60 min. Fused-silica capillary, 50 μm I.D., 50 cm effective length measured to the optical window, with a total length of 57 cm; 14 m*M* sodium borate, 17 m*M* SDS, and 3.5% acetonitrile (pH 9.4), 214 nm, 25°C.

charge on the molecule without a measurable mass difference. Additional research focused on conformational changes in CTLA4Ig monitored by circular dichrosim (CD). In these experiments the CTLA4Ig samples were dissolved in buffers with and without SDS, heated, and then monitored using CE or CD.

The CD spectra in the far-UV region of intact and heat-treated CTLA4Ig with and without SDS were examined to monitor temperature-dependent conformational transformations. Fig. 5A shows the molar ellipticity spectrum of the CTLA4Ig without SDS displaying a negative band at 217 nm, representative of a beta-structure. The spectra demonstrate that the molar ellipticity of CTLA4Ig in the far-UV region was only slightly affected by temperature within the 20–50°C range, indicating the relative stability of CTLA4Ig secondary structure. In a temperature interval between 50 and 55°C, a dramatic alteration of the spectrum occurred, characteristic of a conformational transition. The second region of significant structural transformation was detected between 65 and 75°C with relatively minor structural changes between 55 and 65°C. The results indicate that heat-induced changes of the CTLA4Ig occurred as a two-step process, with a possible relatively stable conformational intermediate in the temperature interval 55–65°C.

Separation of the heat-induced changes in CTLA4Ig-related species without SDS were monitored by CE (Fig. 6A). A three-peak pattern of CTLA4Ig remained unchanged within the temperature range of 25–50°C. Further heating revealed sharp growth of the last migrating species between 65 and 85°C. The first transition between 50 and 55°C observed by CD measure-

Table 2
Summary of CTLA4Ig degradation products separated and collected by ABx and SEC using various stresses

Species by molecular mass	Origin
92–95 000	Intact CTLA4Ig (peaks 1 and 4)
67–64 000	Papain fragment (peak 4); trypsin fragment (peak 1)
56 000	Papain fragment (peak 6)
47 777	Trypsin fragment (peak 1)
36 800	Papain fragment by SEC
30 737	pH 4
27–28 000	Papain fragment (peaks 4, 5, and 6); trypsin fragment (peaks 2 and 3)
25 635	pH 4
11 800–12 000	Papain fragment by SEC; pH 4; trypsin fragment (peak 3)

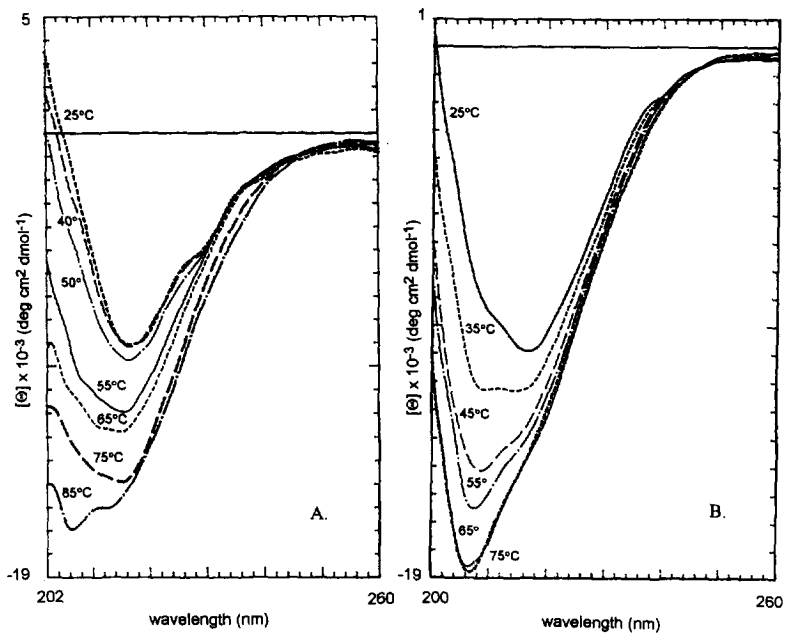

Fig. 5. Temperature dependence of molar ellipticity spectra of CTLA4Ig in the far-UV region: (A) without SDS and (B) in the presence of 1.0 mg/ml SDS. See Experimental section for further details.

ments was not detected by CE, but both methods detected the significant structural transformation between 65 and 85°C.

The presence of SDS in the solution changed the CTLA4Ig CD spectrum at 25°C and facilitated heat-induced transformations. Fig. 5B shows the temperature dependence of CTLA4Ig CD spectra in the presence of 1.0 mg/ml SDS. In this set of experiments, SDS, a known modifier of intramolecular interactions, was used to monitor more closely the parallelism between the CE data changes and the structural transformations. SDS significantly lowers the thermal stability of CTLA4Ig secondary structure. Considerable changes in molar ellipticity are detected at every consecutive temperature increase in the range 25–75°C, suggesting changes in secondary structure. The transition was completed at 65°C, as additional heating to 75°C did not cause further changes in the CD spectra. The parallel experiments monitored by CE are shown in Fig. 6B. The effect of SDS appears to shift the second transition to lower temperatures. The transitions present in the CD spectrum with the presence of SDS, correlate well with the changes observed in the CE electropherograms.

3.3. Rates of degradation of CTLA4Ig under various conditions

As part of the method development studies, the degradation of the CTLA4Ig samples was monitored by CE at specified time intervals. From these timed measurements, the rate at which the reaction proceeds and the apparent kinetics of degradation were determined.

Simple kinetic equations were applied to the degradation data. For a process of C → products, a first-order reaction can be described as [7]:

$$\ln[C] = -kt + \ln[C_0] \quad (1)$$

where [C] is the concentration of C and $[C_0]$ the initial concentration of C, k the rate constant, and t the time of the reaction. For a first-order or pseudo-first-order reaction, a plot of ln [C] vs. t produces a straight line, with a slope of $-k$, the first-order rate constant. The temperature depen-

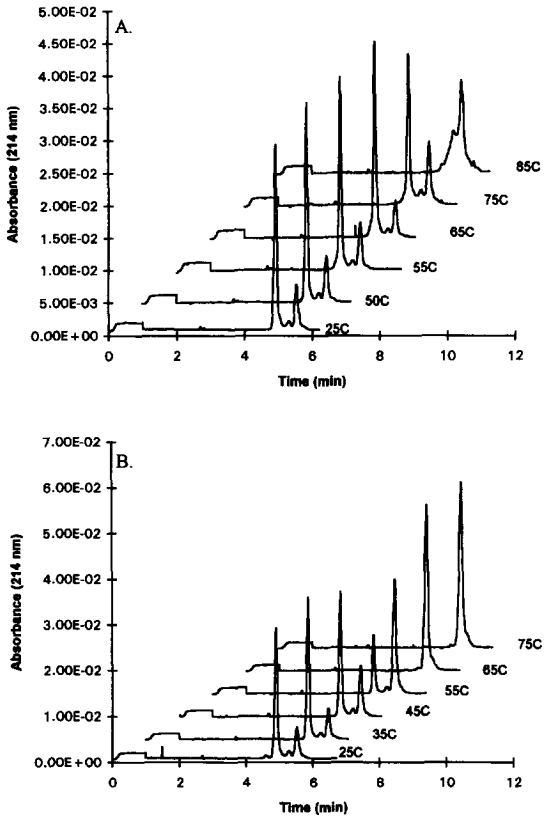

Fig. 6. Heat-induced changes of CTLA4Ig separations: (A) without SDS and (B) in the presence of 1.0 mg/ml SDS. Fused-silica capillary, 75 μm I.D., 50 cm effective length measured to the optical window, with a total length of 57 cm; 14 m*M* sodium borate, 17 m*M* SDS, and 3.5% acetonitrile (pH 9.4), 214 nm, 25°C.

dence of the rate constant can be described by the Arrhenius equation [7]:

$$k = A \exp(-E_a/RT) \quad (2)$$

or

$$\ln k = \frac{-E_a}{RT} + \ln A \quad (3)$$

where E_a is the activation energy, R the gas constant (= 1.987 cal mol^{-1} K^{-1}), T the absolute temperature (K), and A a constant for the reaction. The activation energy can be calculated from the slope of the ln k vs. $1/T$ plot.

The reaction rate constants for four degradation methods were determined by CE, i.e., degradation by H_2O_2, papain, trypsin, and SDS/heat. The change in the height of the first major peak in the electropherogram was monitored with time. First-order reaction kinetics were demonstrated for CTLA4Ig samples treated with papain, H_2O_2, and SDS/heat from a plot of ln ([C]/[C$_0$]) vs. time (in s), see Table 3. Degradation performed with trypsin did not follow common first- or second-order kinetic patterns. Due to the multiple sites of cleavage for trypsin, many peptide fragments were produced which were unresolvable in the CE electropherogram, making measurements in peak height unreliable (Fig. 4A). The relative rates of degradation provided insight into what paths (i.e. sample conditions) are important to maintain the integrity of the molecule.

Fig. 7 shows the conversion of the first peak into the second major peak as a function of time at 50°C in the presence of SDS. It appears that the reaction is a simple A→B conversion (no loss of mass to a third species) since the sum of the heights of the two peaks are constant throughout the measurements. For 50°C the average peak height was $2.92 \cdot 10^{-3}$ with a R.S.D. of 5.5% ($n = 7$), and at 61°C the average peak height was $2.89 \cdot 10^{-3}$ with a R.S.D. of 6.7%

Table 3
Pseudo-first-order rate constants for CTLA4Ig degradation as determined by CE

Degradation method	Equation for line ln ([C]/[C$_0$]) vs. time (s)	k (s^{-1})	r^2
Papain, 25°C	$y = -(8.80 \cdot 10^{-5})x + 0.048$	$8.80 \cdot 10^{-5}$	0.99
Trypsin, 25°C	$y = -(1.88 \cdot 10^{-4})x - 0.45$	$1.88 \cdot 10^{-4}$	0.84
H_2O_2, 25°C	$y = -(3.53 \cdot 10^{-4})x + 0.17$	$3.53 \cdot 10^{-4}$	0.99
SDS, 40°C	$y = -(1.57 \cdot 10^{-4})x + 0.12$	$1.57 \cdot 10^{-4}$	0.99

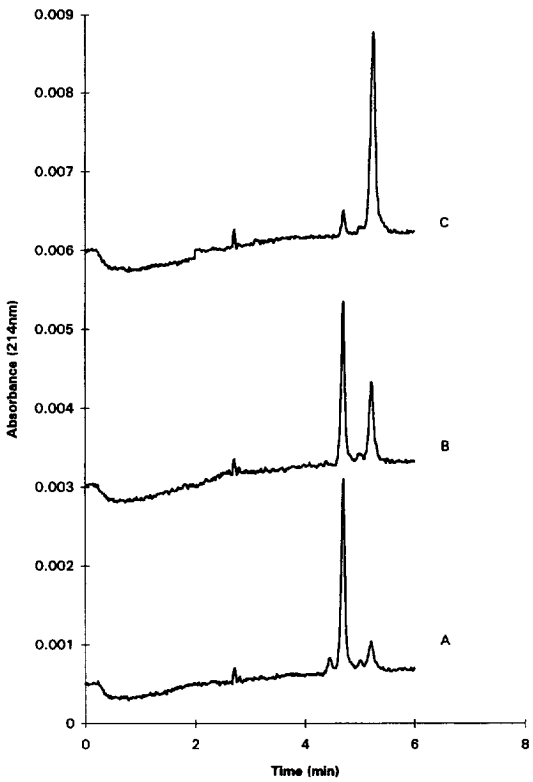

Fig. 7. Conformational monitoring of CTLA4Ig. The sample was diluted with an SDS buffer and then heated to 50°C. The sample was then analyzed at various times: (A) 1 min; (B) 5 min; (C) 30 min. Fused-silica capillary, 75 μm I.D., 50 cm effective length measured to the optical window, with a total length of 57 cm; 14 m*M* sodium borate, 17 m*M* SDS, and 3.5% acetonitrile (pH 9.4), 214 nm, 25°C.

($n = 7$). The kinetic data for the rate of conformational change is displayed in Table 4. An Arrhenius plot, $\ln k$ vs. $1/T$ (T in K), was constructed to calculate the activation energy for the conversion, producing a straight line with an equation of $y = -19300x + 53.3$ with an r^2 of 0.98. The activation energy was calculated to be 38 kcal/mol for the disappearance of the species represented by the first peak in the electropherogram. This value is comparable to other reported literature values for similar transitions. For example, for complete thermal protein unfolding the activation energy is typically on the order of 50 to 100 kcal/mol [8]. Activation energies of 17–22 kcal/mol have been reported for papain, soybean trypsin inhibitor, and lysozyme unfolding on hydrophobic surfaces by reversed-phase HPLC [9]. In a similar manner, the activation energy of unfolding for a recombinant neurotropic factor analyzed by reversed-phase HPLC was 26.2 kcal/mol [10].

The above conformational changes studied by CE allow further characterization of the protein, although the exact structural changes taking place in the molecule are unknown at this time. However, the conformational distribution of species is an important aspect of antibody characterization because only certain conformations may be biologically active. The ability to detect the conformational ratio in a biomolecule becomes important for in-process monitoring. A study into the relative bioactivity of the two main conformers is currently under investigation.

4. Conclusions

The purpose of this study was twofold. The initial work involved developing two LC and one CE procedures that could be used to examine intact and degraded CTLA4Ig samples. The SEC procedure monitored major changes in CTLA4Ig

Table 4
First-order rate constants used to produce Arrhenius plot

Temperature (°C)	Equation for line ln ([C]/[C_0]) vs. time	k (s^{-1})	r^2
30	$y = -(4.95 \cdot 10^{-5})x + 0.14$	$4.95 \cdot 10^{-5}$	0.99
40	$y = -(1.57 \cdot 10^{-4})x + 0.12$	$1.57 \cdot 10^{-4}$	0.99
50	$y = -(1.14 \cdot 10^{-3})x + 0.10$	$1.14 \cdot 10^{-3}$	0.97
61	$y = -(1.24 \cdot 10^{-2})x + 0.73$	$1.24 \cdot 10^{-2}$	0.97
71	$y = -(7.84 \cdot 10^{-2})x + 3.5$	$7.84 \cdot 10^{-2}$	0.97

degradation, and also provided approximate molecular mass values of the principal species. The ABx procedure, although resolving intact protein from similar forms poorly, was quite selective when lower molecular mass species were present. Both methods provided separations that allowed sample collection for further analysis by CE or MS. The CE procedure yielded a high-resolution profile in 20% of the time required for the ABx analysis and required very little material for analysis, with an intra-assay precision of ca. 1%.

Degradation studies followed by LC analysis and sample collection allowed assessment of the selectivity of the separation procedures and provided kinetic data to assess CTLA4Ig solution stability. As expected, use of more than one method (LC and CE) generally yielded a more complete reaction profile. The degradation procedures yielded species in the 12–95 kDa range, as determined by MALDI-TOF–MS. Further characterization of the degradation fragments is currently being undertaken in order to deduce details of the important degradation pathways for the fusion protein.

Conformational transition studies were also performed with the CE (MEKC) procedure. The data provided suggests a simple $A \rightarrow B$ or pseudo $A \rightarrow B$ reaction path. The extent of heat-induced conformational change was found to increase over a factor of 10^3 from 30°C to 71°C, yielding an activation energy of ca. 38 kcal/mol. Thermally induced conformational transitions of CTLA4Ig were also monitored by CD and CE, with reasonable correlation between the two methods. The ability to monitor different conformations in a biomolecule is crucial for process monitoring and bioactivity assessment.

In summary, a combined LC–CE analysis protocol was found to be useful for examining intact and degraded CTLA4Ig. Conformational transition studies by MEKC allowed direct monitoring of the individual conformers and determination of principal kinetic parameters. The relative rates of different kinetic pathways of degradation were also estimated.

The combined approach presented here suggests that LC–CE analysis of intact and degraded proteins of biotechnological interest can yield substantial analytical information that may then be used to monitor the purity of the therapeutic analyte, the presence of impurities, both synthetic and those resulting from degradation, conformational integrity, and relative importance of solution stability pathways.

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