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Development of a peptide mapping procedure to identify and quantify methionine oxidation in recombinant human α1-antitrypsin

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Abstract

A peptide mapping procedure was developed to identify and quantify methionine oxidation in recombinant human α1-antitrypsin. Due to the protein's complex structural biochemistry, chromatographic analysis of methionine containing digest peptides was a significant challenge. However, by using a combination of mass spectrometry, protein engineering, and high-temperature reversed-phase liquid chromatography, we were able to identify methionine residues that are susceptible to oxidation by hydrogen peroxide, and quantify their reactivity. Our results show that five of the protein's 10 methionine residues are susceptible to oxidation at neutral pH, four of which are localized to the active site region. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Peptides; Antitrypsin; Methionine; Hydrogen peroxide

1. Introduction

Human α1-antitrypsin is a M_r 52 000 member of the serpin (serine protease inhibitor) superfamily of plasma proteinase inhibitors [1]. This protein regulates the activity of human neutrophil elastase, a serine protease involved in the degradation of connective tissue components [2]. Oxidation of either methionine 351 (Met351) or methionine 358 (Met358), the protein's two active site methionine residues, results in significant loss of regulatory activity [3]. This loss of regulatory activity in the lungs of smokers is caused by oxidants present in cigarette smoke [4], and contributes to the pathology of pulmonary emphysema [5].

We have chosen to use the recombinant form of human α1-antitrypsin expressed in *E. coli* as a model system for studying protein methionine oxidation during bioprocessing. In order to conduct a quantitative oxidation study, a sensitive analytical technique is required to identify amino acids that are susceptible to oxidation. Analysis of protein primary structure by enzymatic or chemical cleavage [6], and reversed-phase chromatography for the separation of peptide and protein mixtures [7,8], are both well-established techniques. The use of reversed-phase chromatography for separation of peptides generated by enzymatic or chemical cleavage is referred to as peptide mapping. Because peptide mapping is regarded as the key step in analyzing protein primary structure [9], and has a long history in the analysis of biotechnology protein products [10], we chose it as

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our assay to identify and quantify methionine oxidation.

Obtaining a reliable peptide map requires optimization of conditions for both proteolytic digestion and high-performance liquid chromatography (HPLC). Several publications have discussed the difficulties encountered in developing efficient peptide mapping protocols for biotherapeutics [9,11–13]. Such difficulties have been encountered for recombinant human α1-antitrypsin. Although this protein is of interest as a recombinant therapeutic, and for its role in the pathology of several diseases, previously developed mapping procedures have provided only qualitative information [3,14], and no chromatograms have been published.

We have used a combination of liquid chromatography and mass spectrometry (LC–MS) and protein engineering to develop a quantitative peptide mapping protocol for recombinant human α1-antitrypsin. The ability to identify and quantify oxidation via peptide mapping has provided us with a sensitive means of studying protein methionine oxidation during bioprocessing.

2. Materials and methods

2.1. Expression and purification

Recombinant human α1-antitrypsin (α1-AT) was produced in *E.coli* BL21(DE3) harboring the plasmid pEAT8 [15]. Cells were grown on semi-defined media (Na_2HPO_4 42 m*M*, KH_2PO_4 22 m*M*, NaCl 94.6 m*M*, NH_4Cl 18.7 m*M*, $MgSO_4$ 1.0 m*M*, tryptone 10 g/l, glucose 10 g/l, $CaCl_2$ $2H_2O$ 5.2 μ*M*, Na_2EDTA $2H_20$ 81.8 μ*M*, $FeCl_3$ $6H_2O$ 93.8 μ*M*, $CuSO_4$ $5H_2O$ 0.96 μ*M*, $MnSO_4$ H_2O 1.15 μ*M*, $CoCl_2$ $6H_2O$ 1.15 μ*M*, $ZnSO_4$ $7H_2O$ 0.91 μ*M*) containing 100 μg/ml of ampicillin at 37°C. When A_{600} reached 1.4, isopropyl-β-D-thiogalactoside (IPTG) (Roche Molecular Biochemicals, Indianapolis, IN, USA) was added to a final concentration of 0.4 m*M*, and growth was extended for 3 h at 37°C. Cultures were harvested, centrifuged using an IEC CRU-5000 centrifuge (International Equipment Company, Needham Heights, MA, USA) at 2000 *g* for 20 min, and resuspended in 5 ml of 0.1 *M* Tris, 5 m*M* EDTA, pH 8.0 (A_{600}~150). The cells were disrupted in glass tubes using a Branson sonicator with microtip (Branson Ultrasonics, Danbury, CT, USA) at 50% pulsed power, level 3. Each 5 ml aliquot was sonicated in ice water for three 60 s cycles with 30 s cooling between each cycle. Cell extracts were centrifuged using an IEC Centra 4 centrifuge (International Equipment Company) at 14 000 *g* for 20 min and the supernatant was filtered through a 0.22 μm, Millex GS syringe filter (Millipore, Bedford, MA, USA).

The filtered soluble extract (approximately 65 mg) was desalted into 10 m*M* Tris pH 8.0 using PD-10 columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and loaded onto a Q Sepharose high-performance anion-exchange column (3 ml bed volume) (Amersham) in conjunction with a fast protein liquid chromatography (FPLC) system (Amersham) operated at 4°C. After loading, the sample was washed for 7 min with 10 m*M* Tris pH 8.0, followed by elution with a linear gradient from 70 to 140 m*M* NaCl in 35 min. The gradient was held at 140 m*M* NaCl for 15 min to collect fractions containing recombinant α1-antitrypsin. Collected fractions were desalted into Bis–Tris (adjusted to 10 m*M* ionic strength with 37% HCl and titrated to pH 6.3 (4°C)) and loaded onto a Source15 Q PE 4.6/100 anion-exchange column (Amersham) at a flow-rate of 1 ml/min. After loading, the flow-rate was adjusted to 0.75 ml/min. The column was washed for 4 min with starting buffer, followed by a linear gradient from 30 to 60 m*M* NaCl in 33 min. The gradient was held at 60 m*M* NaCl for 10 min to collect fractions containing recombinant α1-antitrypsin. During both chromatographic runs, peak elution was monitored at 280 nm. Purity and yield obtained from each chromatographic step, shown below, were determined by Coomassie Blue stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with densitometric analysis.

Process step	Yield (%)*	Purity (%)
Cell extract	–	4
Q Sepharose high-performance anion-exchange	80	30
Source15 Q PE 4.6/100 anion-change	85	>98

*The recovery yield for each buffer exchange step is approximately 95%, thus resulting in an overall recovery yield of 61%.

2.2. Site-directed mutagenesis

Site-directed mutants were constructed using the following primers with the plasmid pEAT8 as a template:

C232S forward (5′ to 3′)	GGC ATG TTT AAC ATC CAG CAC TCT AAG AAG CTG TCC AGC TGG
C232S reverse (5′ to 3′)	CCA GCT GGA CAG CTT CTT AGA GTG CTG GAT GTT AAA CAT GCC
M351V forward (5′ to 3′)	GGG ACT GAA GCT GCT *GGA GCG* GTG TTT TTA GAG GCC ATA CC
M351V reverse (5′ to 3′)	GG TAT GGC CTC TAA AAA CAC *CGC TCC* AGC AGC TTC AGT CCC
M358V forward (5′ to 3′)	G GCC ATG TTT TTA GAG GCC ATA *CCG* GTA *TCG* ATC CCC CCC GAG G
M358V reverse (5′ to 3′)	C CTC GGG GGG GAT *CGA* TAC *CGG* TAT GGC CTC TAA AAA CAT GGC C

Underlined codons indicate sites of point mutations for the desired amino acid change, and italicized codons indicate silent mutation sites for identification of successful mutagenesis by restriction enzyme digestion. All custom primers were obtained with cartridge purity from Invitrogen (Carlsbad, CA, USA). M351V, M358V and C232S mutants were constructed using the Quikchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

2.3. In vitro oxidation for peptide mapping

Purified recombinant α1-AT was desalted into 10 m*M* phosphate, pH 7.0 (ionic strength adjusted to 0.1 *M* with NaCl) and equilibrated at 25°C (unless otherwise noted). Oxidation reactions were performed with various concentrations of hydrogen peroxide by dilution of a stock of 30% (w/w) H_2O_2 (Sigma, St. Louis, MO, USA). At various times after oxidation was initiated, samples were removed and desalted using PD-10 columns (Amersham). Desalt columns were equilibrated with endoproteinase Lys-C digestion buffer [25 m*M* Tris–HCl pH 8.5, 1 m*M* EDTA, 10% (v/v) HPLC grade acetonitrile (EM Science, Gibbstown, NJ, USA)].

2.4. Protein digestion for peptide mapping

Desalted samples were incubated with 7.5% (w/w) endoproteinase Lys-C (EC 3.4.21.50, Roche Molecular Biochemicals, Indianapolis, IN, USA) for 25 h at 37°C in a model PTC-150 thermal cycler (MJ Research, Waltham, MA, USA). Following digestion, the acetonitrile present in the digestion buffer was removed by SpeedVac rotary vacuum centrifugation (Savant Instruments, Holbrook, NY, USA) at room temperature for 60 min. Samples were stored at −20°C until further analysis.

2.5. Analytical HPLC

For routine analytical runs, Lys-C peptides generated from 10 to 13 μg of recombinant α1-AT were injected onto a 3.2×250 mm reversed-phase LC column, model 218MS53 (Vydac, Hesperia, CA, USA), using a model AS-4000 autosampler (Hitachi Instruments, San Jose, CA). Column temperature was controlled with an Eppendorf TC-45 column heater (Brinkmann Instruments, Westbury, NY, USA). Peak areas were integrated using System Gold Nouveau software (Beckman Coulter, Fullerton, CA, USA) with data collected from the HPLC system consisting of a 168 diode array detector and 126 pumps (Beckman Coulter). All runs were performed with HPLC-grade water and ACN purchased from EM Science, and 99+% spectrophotometric grade trifluoroacetic acid (TFA) (Aldrich, Milwaukee, WI, USA).

2.6. LC–MS

The elution pattern of native and oxidized digest peptides was determined using LC–MS. Runs were performed on a Perkin–Elmer Sciex Model 365 triple stage mass spectrometer (Perkin Elmer Ana-

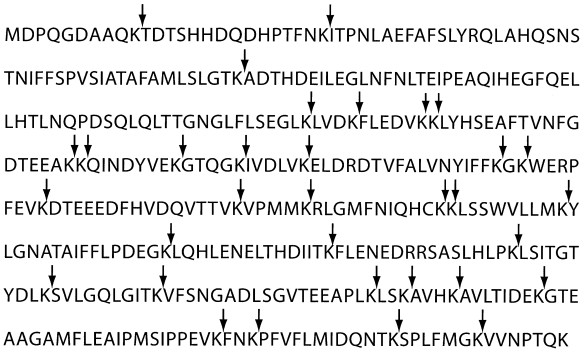

Fig. 1. Recombinant α1-antitrypsin's linear amino acid sequence (single letter abbreviations). Arrows indicate endoproteinase Lys-C cleavage sites.

lytical Instruments, Norwalk, CT, USA) attached to an Applied Biosystems Model 140 C HPLC system (Applied Biosystems, Foster City, CA, USA). Peptides from Lys-C digestion of 2 μg of recombinant α1-AT were injected onto a 1.0×250 mm reversed-phase LC column, model 218MS51 (Vydac, Hesperia, CA, USA), operated at a flow-rate of 50 μl/min. The column eluent flowed directly into the electrospray interface, and mass spectra were acquired over an m/z range of 500–1800.

3. Results and discussion

3.1. Reaction conditions and selection of protease

The objective of peptide mapping α1-antitrypsin was to identify methionine residues that are susceptible to oxidation at neutral pH. We chose to study methionine oxidation at pH 7 because this is approximately the pH at which the protein normally functions in human blood plasma, and is a pH at which the protein has high stability against aggregation [16,17]. As expected, we did not observe aggregation or precipitation during any experimental work performed at pH 7.

Recombinant α1-antitrypsin contains 10 methionine residues widely spaced throughout its primary sequence (Fig. 1). Therefore, fragmentation sufficient to resolve the reduced and oxidized forms of each is a difficult task. Only two enzymes, trypsin and Lys-C, are theoretically capable of providing an acceptable digest profile. Because α1-antitrypsin is an inhibitor of trypsin, Lys-C was the enzyme chosen for digestion. The peptides generated by Lys-C cleavage of α1-antitrypsin are shown in Fig. 1, and those containing methionine are listed in Table 1.

3.2. Chromatographic separation and peptide analysis

3.2.1. LC–MS

The temperature and buffer used for proteolytic digestion were optimized to achieve complete digestion in 25 h (as determined by mass spectrometry of digest peptides). Once digestion was complete, reversed-phase chromatography was used for peptide separation, and mass spectrometry was used for analytical characterization. Because an α1-antitrypsin digest can theoretically include up to 24 peptides containing methionine and methionine sulfoxide, off-line analysis would have been very dif-

Table 1
Methionine containing peptides generated from Lys-C digestion of recombinant α1-antitrypsin

Peptide	Sequence position	Peptide sequence	Observed molecular mass (predicted)
L1	1–10	MDPQGDAAQK	1059.8 (1059.5)
L3	26–69	ITPNLAEFASLYRQLAHQSNSTNIFFSPVSIATAFAMLSLGTK	4807.4 (4806.5)
L17	218–222	VPMMK	604.4 (604.3)
L18	223–233	RLGMFNIQHCK	1345.9 (1345.7)
L20	235–243	LSSWVLLMK	1075.9 (1075.6)
L30	344–365	GTEAAGAMFLEAIPMSIPPEVK	2259.3 (2259.6)
L32	369–380	PFVFLMI DQNTK	1452.8 (1450.9)
L33	381–387	SPLFMGK	778.4 (778.4)

Predicted peptide masses (shown in parenthesis) are listed next to those that were experimentally determined.

ficult and time consuming. Therefore, we chose liquid chromatography coupled with on-line electrospray mass spectrometry (LC–MS) for rapid development of a peptide map in which all methionine containing peptides could be identified.

Chromatography was performed using a Vydac low TFA column. This column reduces the separation dependence on ion-pair reagents to the extent that 0.01% TFA can be used in mobile phase buffers while maintaining sharp, symmetrical chromatographic peaks. The low TFA concentration allowed us to avoid post column additive techniques or large quantities of digest protein to overcome the suppressive effect on ion generation of higher TFA concentrations [18]. We found that 0.01% TFA was sufficient to resolve most of the digest peptides, and determine their relative elution order. However, it was eventually necessary to increase the TFA concentration to 0.03% to resolve all methionine and methionine sulfoxide-containing peptides for quantitative purposes.

3.2.2. Active site peptide chromatography

Structural studies of human [19] and recombinant human α1-antitrypsin [3] have shown that the protein's two active site methionine residues, Met351 and Met358, are highly susceptible to oxidation. Therefore, it is critical to be able to identify and quantify the reduced and oxidized forms of the peptide containing these residues (L30, Table 1). Although identifiable by LC–MS, the active site peptide eluted from the reversed-phase column as a broad peak with significant tailing during chromatographic separation at room temperature. Because quantification is as important as identification, conditions had to be established that would reduce or eliminate the severe tailing. The broadening and splitting of reversed-phase HPLC peaks as a result of slow *cis–trans* peptide bond isomerization in proline-containing dipeptides has been studied in detail by Melander et al. [20]. Several other authors have reported similar chromatographic behavior in longer proline-containing peptides, but only those with one or more proline–proline (Pro–Pro) peptide bonds [13,21,22]. The active site peptide is long (22 amino acids), and contains a Pro–Pro sequence (Pro361–Pro362). Therefore, the possibility that Pro361–Pro362 peptide bond isomerization was responsible for the chromatographic profile of the active site peptide was investigated.

It is well established that a steep gradient slope or high column operating temperature can be used to enhance separation efficiency during reversed-phase chromatography of peptides that contain one or more slowly isomerizing proline peptide bonds [20,22]. Because a shallow gradient slope is necessary for resolution of methionine containing peptides in an α1-antitrypsin digest, we chose to investigate column temperature as a means of improving chromatography of the active site peptide.

With an activation energy of 22 kcal/mol [23], the rate of Pro–Pro *cis–trans* isomerization is approximately 154 times faster at 65°C than it is at 20°C (1 cal=4.184 J). Therefore, we were able to use high-temperature chromatography (65°C) to increase the isomerization rate of the active site peptide's Pro361–Pro362 peptide bond, and thus eliminate distinct populations of isomerizing conformers on the chromatographic timescale. Because high-temperature chromatography resulted in the elution of a symmetric, quantifiable peptide (Fig. 2), we chose to perform peptide separations at 65°C to maximize column efficiency and achieve a sharp, clean L30 elution profile.

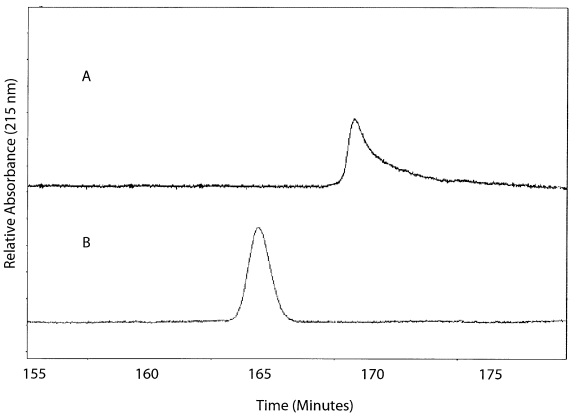

Fig. 2. Elution profile of the active site peptide (L30) (A) at 20°C and (B) at 65°C. A gradient from Buffer A (0.03% TFA) to B (50% ACN, 0.0285% TFA) was run as follows: 0% from 0 to 4 min, 0–56% from 4 to 170 min, 56–88% from 170 to 210 min, and 88–100% B from 210 to 215 min.

3.3. Analytical characterization with peptide mapping

3.3.1. Identification of oxidized and reduced methionine containing peptides

Chromatographic separation at 65°C resolved the peptide digest fragments containing one or more of the protein's 10 methionine residues. In vitro peptide oxidation followed by LC–MS was used to generate a reference peptide map containing native peptides as well as those with methionine residues oxidized to methionine sulfoxide.

One problem that we encountered, however, was extremely rapid oxidation of the peptide containing cysteine 232 (Cys232) and methionine 226 (Met226) (L18, Table 1). The rate at which the reduced form of this peptide was modified by hydrogen peroxide at neutral pH was much too fast to be attributable to Met226 oxidation. Therefore, we reasoned that the observed modification was Cys232 oxidation. Fig. 3 shows a chromatographic profile for the initial oxidation product of peptide L18 generated by digestion of α1-antitrypsin that had been exposed to *tert*.-butyl hydrogen peroxide (TBHP), a much less reactive oxidant than hydrogen peroxide, at 4°C.

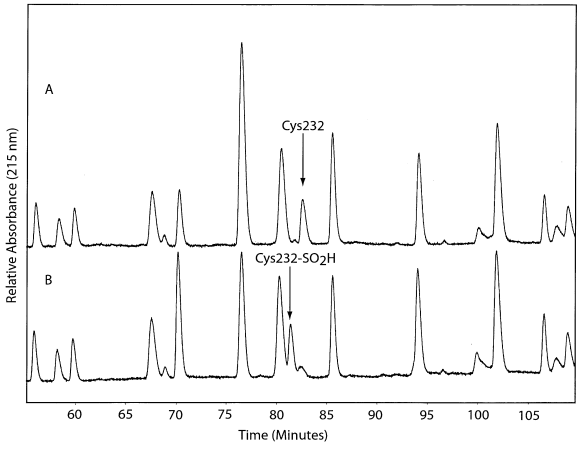

Fig. 3. Peptide map of recombinant α1-antitrypsin (A) prior to exposure to oxidant and (B) after 40 min exposure to 1 m*M* *tert*.-butyl hydrogen peroxide in 10 m*M* phosphate, pH 7.0 at 4°C. The reduced form of the peptide containing cysteine 232 (L18) is denoted Cys232, and the oxidized form is denoted Cys232–SO_2H. Twelve μg of recombinant α1-antitrypsin was applied to a Vydac 218MS53 (3.2×250 mm) column. A gradient from Buffer A (2.5% ACN, 0.01% TFA) to B (65% ACN, 0.008% TFA) was run as follows: 0% from 0 to 4 min, 0–15.5% from 4 to 67 min, 15.5–19% from 67 to 88 min, and 19–100% B from 88 to 210 min. The flow-rate was 0.5 ml/min and the column temperature was 20°C.

The rapidly formed L18 oxidation product shown in Fig. 3 has a mass of +32. Three other oxidized variants of this peptide are generated following extended exposure to hydrogen peroxide or TBHP. Two of these species have a mass of +48, and the third +64. In contrast, only a +16 L18 variant is observed when a cysteine 232 to serine mutant (C232S) is exposed to hydrogen peroxide, and this variant is only observed in digests of protein that has been extensively oxidized. Therefore, the +32 mass increase for the rapidly formed oxidation product shown in Fig. 3 corresponds to the formation of Cys232 sulfinic acid (Cys–SO_2H). The following combinations of Cys232 and Met226 oxidization account for the observed +48 and +64 L18 variants; Cys–SO_2H (+32), Cys–SO_2H/Met226SO (+48), Cys–SO_3H (+48), and Cys–SO_3H/Met226SO (+64). Although sulfinic and cysteic acids were the only Cys232 oxidation products found with peptide mapping, it should be noted that these are stable oxidation states that follow the formation of sulfenic acid. A stable sulfenic acid intermediate is only possible when the structural environment surrounding it has limited solvent access and contains amino acids capable of providing hydrogen bond stabilization [24]. Therefore, the acid forms of Cys232 found with peptide mapping may not be the actual oxidation states of Cys232 prior to proteolytic digestion; other techniques for studying Cys232 oxidation are being developed.

Because Cys232 oxidation occurs approximately 1000 times faster than Met226 oxidation at neutral pH, peptides with oxidized Met226 were only observed in conjunction with the sulfinic and cysteic acid forms of Cys232. This made it very difficult to independently assess the susceptibility of Met226 to oxidation. Therefore, a C232S mutant was used in oxidation studies to limit the scope of our present work to methionine oxidation. In agreement with prior work [25], we found that this mutation does not affect protein conformation or stability. Peptide maps of C232S exposed to hydrogen peroxide in vitro are shown in Fig. 4.

Peptides that contain methionine sulfoxide are expected to elute at earlier retention times during

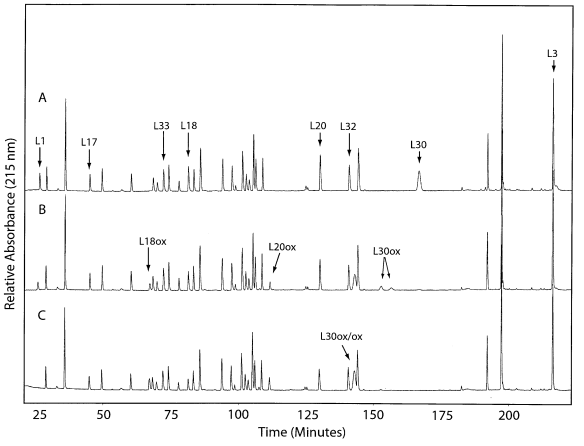

Fig. 4. Peptide maps of (A) C232S, (B) C232S oxidized 1.5 h, and (C) C232S oxidized 5.5 h with 20 m*M* H_2O_2 in 10 m*M* phosphate, pH 7.0 at 25°C. A gradient from Buffer A (0.03% TFA) to B (50% ACN, 0.0285% TFA) was run as follows: 0% from 0 to 4 min, 0–56% from 4 to 170 min, 56–88% from 170 to 210 min, and 88–100% B from 210 to 215 min. The flow-rate was 0.5 ml/min and the column temperature was set at 65°C. The active site peptide (L30) has three oxidized forms. Those oxidized only at Met351 or Met358 are labeled L30ox, while the peptide containing both oxidized Met351 and oxidized Met358 is labeled L30ox/ox. The identity of the mono-oxidized species is discussed in the text.

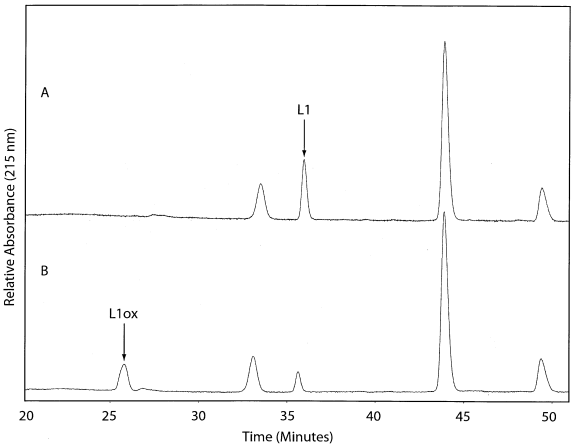

Fig. 5. Low temperature chromatography of (A) reduced L1 peptide and (B) L1 peptide generated by oxidation of α1-antitrypsin for 90 min with 30 m*M* H_2O_2 in 10 m*M* phosphate, pH 7.0 at 25°C. A gradient from Buffer A (0.01% TFA) to B (50% ACN, 0.009% TFA) was run as follows: 0% from 0 to 4 min, 0–17.5% from 4 to 62 min. The flow-rate was 0.5 ml/min and the column temperature was 20°C.

reversed-phase chromatography than their reduced counterparts. Therefore, it is clear from analysis of Fig. 4 that Met1, Met226, Met242, Met351, and Met358 are susceptible to oxidation by hydrogen peroxide at neutral pH. The peptide map allowed us to clearly identify, but not completely quantify, the reactivity of these five methionine residues due to variable recovery of the peptide containing Met1 (L1), and the presence of two mono-oxidized forms of the peptide containing Met351 and Met358 (L30). In order to quantify these peptides, we had to determine the cause of the variable L1 recovery, and the identity of the mono-oxidized forms of L30.

3.3.2. Analysis of L1

Recombinant α1-antitrypsin peptide maps showed variable recovery of the peptide containing Met1 (L1, Table 1) during high-temperature peptide separations. Since the most reactive methionine residues, Met351 and Met358, were not oxidized, it was unlikely that this was the result of methionine oxidation prior to, or during chromatography. The complete recovery of the L1 peptide at 20°C (Fig. 5), however, made it clear that some type of temperature-dependent degradation reaction, other than oxidation, was occurring on the reversed-phase column.

The N-terminus of recombinant α1-antitrypsin derived from the plasmid pEAT8 begins with the amino acid sequence Met–Asp–Pro. Cleavage at aspartic acid–proline (Asp–Pro) peptide bonds under acidic conditions at elevated temperature is well documented [26–28]. We used LC–MS to determine whether this cleavage occurs at α1-antitrypsin's Asp2–Pro3 peptide bond during high-temperature reversed-phase separation of the native protein. Because α1-antitrypsin's N-terminus is undefined by X-ray data and has known susceptibility to proteolytic cleavage [29], we expected that it would be exposed and flexible in the native structure, and thus susceptible to the same reactions as the L1 peptide during reversed-phase chromatography.

In agreement with this theory, approximately 5% of the protein is hydrolyzed at the Asp2–Pro3 peptide bond during separations in 4 m*M* ammonium acetate buffer, pH 3.0 when operating at a column temperature of 70°C. Because the activation energy for Asp–Pro peptide bond hydrolysis is approximate-

ly 19 kcal/mol [30], we were able to reduce this cleavage to less than 1% by lowering the column temperature to 50°C (Fig. 6).

Since the L1 peptide is very hydrophilic, a variant hydrolyzed at the Asp2–Pro3 bond is not retained on the reversed-phase resin at high temperature. Therefore, we have not observed an L1 variant beginning with Pro3 during peptide separations. However, the low pH (pH 3 at 0.01% TFA) and elevated temperature used in our peptide mapping protocol are nearly identical to the conditions responsible for Asp–Pro cleavage in the native protein.

Because high temperature significantly improves chromatography of the active site peptide, and this peptide is critical in our study of methionine oxidation, we perform reversed-phase peptide separations at both 65°C and 20°C. The higher temperature is used for active site quantification, while the lower temperature is best for N-terminal analysis.

3.3.3. Analysis of L30ox

Three forms of the active site peptide were observed following in vitro oxidation. The most hydrophilic of these was oxidized at both Met351

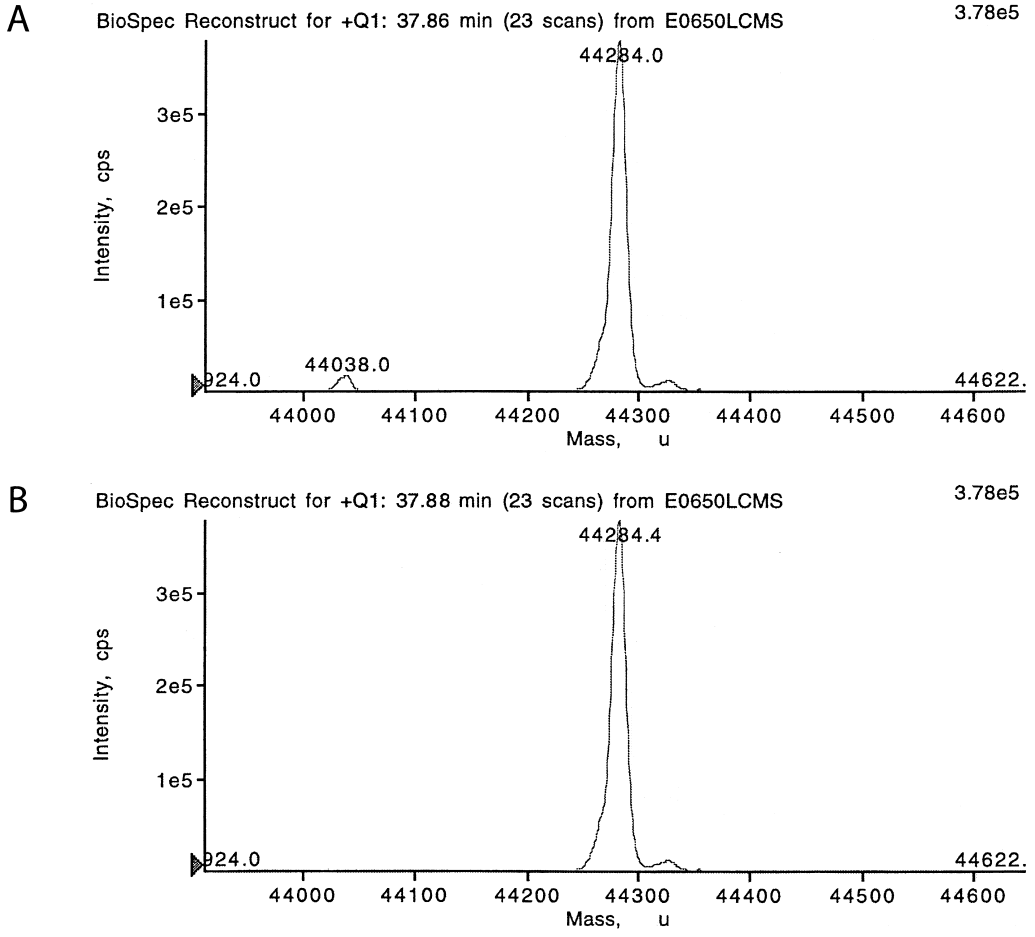

Fig. 6. Deconvoluted molecular mass spectrum of purified recombinant α1-antitrypsin (C232S) eluting on a reversed-phase gradient with the column temperature set at either (A) 70°C or (B) 50°C. The peak at 44 038 in (A) corresponds to protein that has been hydrolyzed at the Asp2–Pro3 peptide bond. 35 pmol of purified protein was loaded for each run, and the gradient from Buffer A (4 m*M* ammonium acetate, 0.1% formic acid) to B (4 m*M* ammonium acetate, 0.085% formic acid) was as follows: 5–100% from 0 to 45 min, 100% B from 45 to 60 min. The flow-rate was 40 μl/min and mass spectra were acquired over an m/z range of 700–2300.

and Met358 (+32). The next two had masses corresponding to the active site peptide plus an additional oxygen atom (+16). Because retention time during reversed-phase chromatography is sequence dependent for some peptides [31], the two +16 active site peptides could have been different mono-oxidized forms, one oxidized exclusively at Met351 and the other oxidized exclusively at Met358. An alternative explanation was that oxidation at Met351 was not resolved from oxidation at Met358, and these peaks were a result of the different chromatographic retention times sometimes observed for *R* and *S* chiral configurations of methionine sulfoxide containing peptides [32].

Cyanogen bromide cleavage is commonly used to determine the identity of oxidized methionine in polypeptides with more than one methionine residue. However, the results of this technique can be difficult to interpret [3]. Therefore, we chose to make mutants with methionine to valine substitutions at Met351 (M351V) and Met358 (M358V) to determine the identity of the oxidized active site peptides.

The chromatograms shown in Fig. 7 indicate that mono-oxidized forms of the active site peptide are resolved due to a difference in the location of methionine sulfoxide within the active site peptide sequence, and not because of methionine sulfoxide chirality. When the oxidized forms of Met351 (M358V) and Met358 (M351V) are compared to the L30ox peptides shown in Fig. 4, it is clear that the L30 peptide with oxidized Met351 is readily resolved from the earlier eluting L30 peptide containing oxidized Met358.

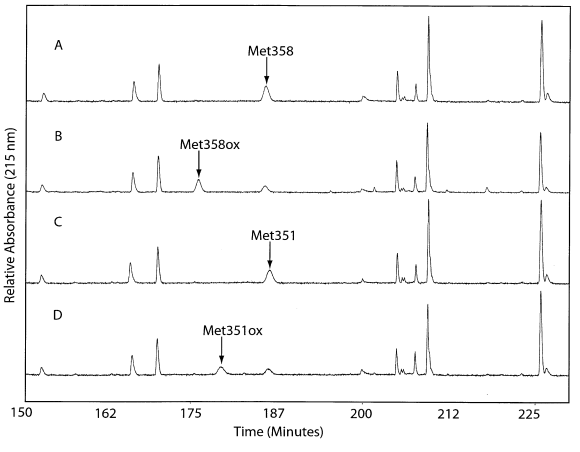

Fig. 7. Identification of oxidized Met351 and Met358 peptides. Peptide maps of (A) M351V, (B) oxidized M351V, (C) M358V, and (D) oxidized M358V. Oxidation reactions were carried out with 30 m*M* H_2O_2 for 45 min in 10 m*M* phosphate, pH 7.0 at 25°C.

3.3.4. Quantification of methionine oxidation

Identification of the peptides corresponding to oxidized Met351 and oxidized Met358, combined with peptide separations at 20°C and 65°C, allowed us to quantify the reactivity of recombinant α1-antitrypsin's methionine residues (Fig. 8).

The reactivity of Met1 is intuitive on the basis of the commonly encountered reactivity of N-terminal methionine residues [33,34], and the reactivity of both Met351 and Met358 is expected due to their locations in α1-antitrypsin's exposed active site loop. However, the structural factors governing the oxidative susceptibility of Met226 and Met242 are less clear. These residues are located near Met351 and Met358 in the protein's active site region, but different high-resolution crystal structures depict varying degrees of side-chain solvent exposure [35,36]. Further work is required to determine whether conformational flexibility determines reactivity in and around the active site loop, and whether

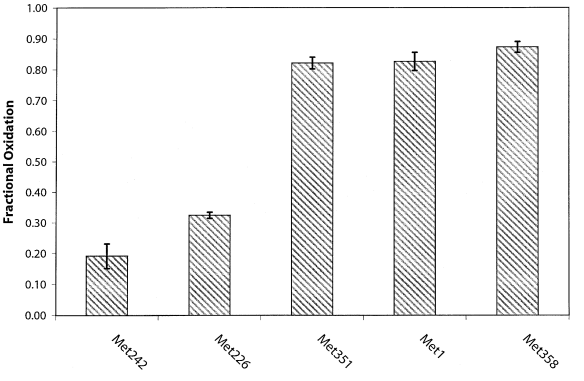

Fig. 8. Reactivity of recombinant α1-antitrypsin's methionine residues. In vitro oxidation was carried out with 20 m*M* H_2O_2 in 10 m*M* phosphate, pH 7.0 for 150 min at 25°C. The fractional oxidation of each methionine was calculated by dividing the area of the peptide containing the oxidized form of a particular methionine residue by the combined areas of the peptides containing the reduced and oxidized forms of that residue. Error bars represent the SD of five separate experiments performed under identical conditions.

such flexibility can be manipulated via bioprocessing environment.

4. Conclusion

We have used a combination of LC–MS, protein engineering, and high-temperature reversed-phase chromatography to develop and characterize a peptide map for recombinant human α1-antitrypsin. Peptide mapping allowed us to conclusively identify the five methionine residues in recombinant α1-antitrypsin's primary structure that are susceptible to oxidation by hydrogen peroxide at neutral pH; Met1, Met226, Met242, Met351, and Met358. These results are consistent with, and extend the work of, Carp et al. [37] in which four unidentified residues of methionine sulfoxide were found in human α1-antitrypsin isolated from bronchoalveolar lavage fluid of smokers (human α1-antitrypsin does not contain Met1). Taggart et al. [3] determined that recombinant α1-antitrypsin's Met226, Met351, and Met358 are susceptible to oxidation by hydrogen peroxide, but mentioned neither the susceptibility of Met242 to oxidation, nor the difference in reactivity between Met351 and Met358. They, however, conducted their experiments at pH 5.0, and did not base their quantitative results on peptide mapping. In addressing the first issue, it is possible that recombinant α1-antitrypsin adopts different conformations at pH 5.0 and pH 7.0, resulting in different methionine oxidation profiles. It has been shown that recombinant α1-antitrypsin aggregates approximately 65 times faster at pH 5 that at pH 7 [16]. The same pH-dependent conformational changes that affect aggregation may also affect methionine oxidation. With regards to the analytical procedures used to determine methionine oxidation, the simultaneous peptide sequencing method used by Taggart et al. differs from peptide mapping. Therefore, it is difficult to determine whether the discrepancy in results can be attributed to analytical methods.

The peptide mapping technique described here provides us with an analytical tool for studying methionine oxidation in recombinant α1-antitrypsin during bioprocessing. However, the rapid oxidation of Cys232 found in this study indicates that other potential routes of oxidative degradation also need to be addressed using different analytical techniques.

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