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Analysis and sequencing of the active-site peptide from native and organophosphate-inactivated acetylcholinesterase by electrospray ionization, quadrupole/time-of-flight (QTOF) mass spectrometry

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Abstract

A method to identify and sequence recombinant mouse acetylcholinesterase (rMoAChE) including the native and organophosphate-modified active-site peptides was developed using capillary liquid chromatography with electrospray ionization, quadrupole/time-of-flight mass spectrometry. Addition of 2-propanol to the reversed-phase gradient system and a decreased gradient slope improved the peptide resolution and the signal of the active-site peptide. The highest protein coverage and active-site peptide signal were achieved when the rMoAChE:chymotrypsin ratio of 5:1 was used with digestion at 37 °C. rMoAChE and the active-site peptide were identified and sequenced from chymotryptic digests of native, methyl paraoxon-, and ethyl paraoxon-inactivated rMoAChE showing unequivocally that the exact modification site was the active-site serine.

Keywords: Acetylcholinesterase; QTOF; MS; Active site; Peptide; Chymotrypsin; Organophosphate; Methyl paraoxon; Ethyl paraoxon

1. Introduction

Acetylcholinesterase (AChE) belongs to a large family of serine hydrolases [1] whose specific biological role is to terminate the neuronal impulse that occurs when acetylcholine (ACh) is released into the synaptic cleft. There are several different polymorphic forms of AChE within a species, yet the catalytic domains, enzyme mechanism and pharmacology are nearly identical [2]. *Torpedo californica*, *mus musculus*, and *homo sapiens* AChE have been characterized by X-ray analysis [3–5] and reveal the typical serine hydrolase catalytic triad (Glu-Ser-His). AChE structure is also defined by a 20 Å gorge lined with lipophilic residues [6] that connect the active-site region to the protein surface. A high degree of sequence homology exists among species and results in a conserved three-dimensional active-site structure whose mechanism is hinged upon a highly nucleophilic serine hydroxyl group. The serine hydroxyl reacts with the ACh ester carbonyl group to form the transiently acetylated AChE that is hydrolyzed by water to afford acetic acid and restored AChE. AChE maintains the correct titers of ACh in the synapse to ensure proper neuronal signaling and nerve cell health. Inactivation or loss of AChE is a serious biochemical consequence resulting in an accumulation of ACh in cholinergic synapses and subsequent hyperstimulation of skeletal muscle, smooth muscle, and secretory glands, altered cardiac activity, respiratory compromise and, in extreme cases, death [7].

Organophosphate (OP) esters are a diverse class of compounds that include oxidation products of insecticides (e.g., malaoxon from malathion, paraoxon from parathion), protease inhibitors (e.g., diisopropyl fluorophosphates), and chemical warfare agents (e.g., sarin, soman, VX). Common to all OPs are the acute toxic effects, which result from reaction with an active-site serine hydroxyl group of AChE to form a stable

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; rMoAChE, recombinant mouse acetylcholinesterase; anti-mAChE, anti-mouse AChE antibody; $AChE_{10S}$, TLFGESAGAA-COOH; $AChE_{14S}$, GESAGAASVGMHIL-COOH; HFIP, hexafluoroisopropanol; BSA, bovine serum albumin; PBS, phosphate buffered saline; Tween-20, poloxyethylene 20 sorbitan monolaurate; DMSO, dimethylsulfoxide; GFP, [Glu^1]-fibrinopeptide B; ESI, electrospray ionization; QTOF, quadrupole/time-of-flight; MS, mass spectrometer, mass spectrometry; CID, collision-induced dissociation; EIC, extracted ion chromatogram; OP, organophosphate

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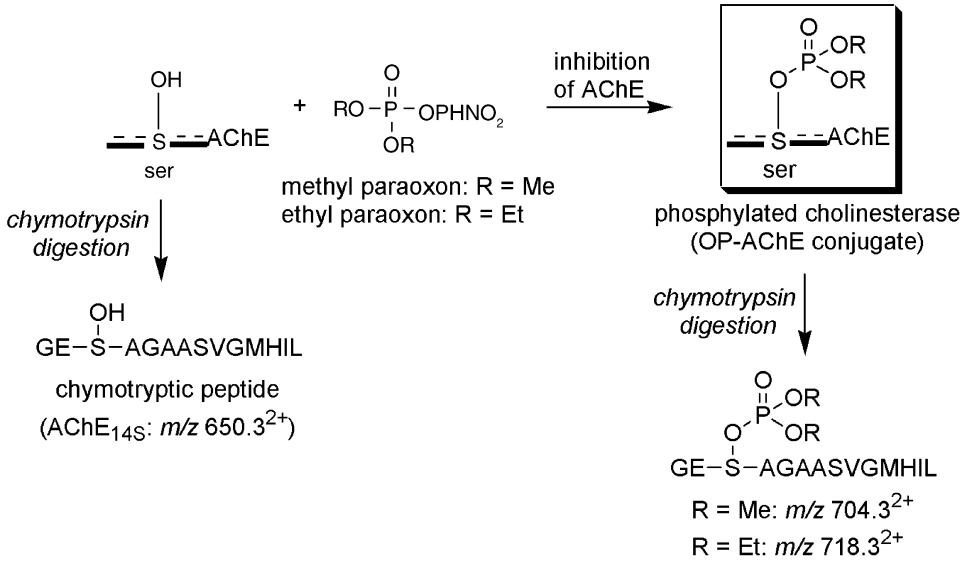

Fig. 1. Depiction of AChE, the reaction with methyl paraoxon and ethyl paraoxon, and formation of the active-site-containing peptides following chymotrypsin digest (doubly charged ion values shown).

phosphoserine ester bond or an OP-AChE conjugate (Fig. 1; shown for paraoxon), rendering the enzyme inactive and unable to hydrolyze ACh. The mechanism of inactivation typically occurs with loss of a leaving group from the OP compound, for example, a *p*-nitrophenoxy leaving group is ejected when methyl or ethyl paraoxon inhibits AChE (Fig. 1). A number of studies have been undertaken to understand the chemical nature of the OP-AChE modification [3,8–14]. Importantly, OP-AChE conjugates differ in the phosphoester ligands, which defines a unique molecular weight identifier for analysis and potentially a method for discriminating between OP-AChE conjugates.

Mass spectrometry offers a rapid method to characterize proteins and their modifications. Certain OP-modified AChEs have been characterized by ESI-quadrupole MS [8,9,15] and matrix-assisted laser desorption ionization (MALDI)-TOF MS [10–12]. Although these studies established that an OP group was added to AChE, the exact site of modification and sequence information could not be unambiguously determined. In one case, the chemical structure of the OP-peptide conjugate could not be obtained without the use of isotopic labeling, due to limited mass accuracy [11]. Although these prior studies demonstrated how mass spectrometry can differentiate OP-modified AChE from native AChE, the need to determine the mechanism of OP-AChE conjugate formation using high-resolution mass spectrometric sequence analysis remains.

The recombinant enzyme, rMoAChE [16], was selected for study owing to the large number of protein impurities found in commercial sources of electric eel and bovine erythrocyte AChE. Further, rMoAChE and the active-site peptides obtained from chymotryptic digests (Fig. 1) are more than 90% homologous to human AChE and its corresponding peptide fragments. Therefore, MS methods developed for rMoAChE will be applicable to other mammalian AChEs, and other select esterases (e.g., butyrylcholinesterase) bearing homologous active-site sequences. To further aid the analysis, the active-site serine inclusive peptides TLFGESAGAA ($AChE_{10S}$) [14] and GE*S*AGAASVGMHIL ($AChE_{14S}$; equivalent to the chymotryptic fragment, Fig. 1), were synthesized and used to develop methods for detection of the active-site peptide from rMoAChE. Using rMoAChE, $AChE_{10S}$, and $AChE_{14S}$, we report a new method that identifies and sequences native, methyl paraoxon-inactivated, and ethyl paraoxon-inactivated rMoAChE. Because an overwhelming majority of the current OP insecticides contain either dimethoxy or diethoxy groups (Fig. 1), the ability to identify and discriminate between these OP-AChE conjugates and native AChE by tandem MS will have broad application.

2. Experimental

2.1. Materials

HPLC-grade water, acetonitrile, 2-propanol, and Micron YM-10^{TM} filters (Micron Bioseparations) were purchased from Fisher Scientific. Chymotrypsin, thrice crystallized and treated with 1-chloro-3-tosylamido-7-amino-2-heptanone to inhibit trypsin activity, was purchased from Worthington Biochemical Corp (Lakewood, NJ). The synthetic peptides TLFGE*S*AGAA-CO_2H ($AChE_{10S}$) and GE*S*AGAASVGMHIL-CO_2H ($AChE_{14S}$) were commercially synthesized by Synpep (Dublin, CA). Human [Glu^1]-fibrinopeptide B (GFP) used for MS calibration and optimization of ESI conditions was purchased from Sigma–Aldrich. rMoAChE was prepared and purified as described [16]. Methyl paraoxon and ethyl paraoxon were purchased from ChemService Inc. (West Chester, PA).

2.2. Capillary chromatography conditions

Analysis of peptide standards and the separation and analysis of the proteolytic peptides were achieved on a Waters

Table 1
Capillary LC solvent gradients used for analysis of chymotryptic digests of rMoAChE

Solvent A (%)	Solvent B (%)	Time (min)
Gradient 1		
95	5	0–3
50	50	15
20	80	16–20
Gradient 2		
95	5	0–3
60	40	60
20	80	70

Solvent A: 0.2% formic acid in water:acetonitrile (98:2, v/v). Solvent B: 0.2% formic acid in acetonitrile, 0.2% formic acid in DMSO:acetonitrile (10:90, v/v), or 0.2% formic acid in 2-propanol:acetonitrile(10:90, v/v).

CapLC coupled to a QTOF-I MS. GFP or peptide standards (4 μL of a 0.5 pmol μL^{-1} solution) were injected followed by a 10 μL injection of rMoAChE chymotryptic digest (0.2 pmol μL^{-1}), through an auxiliary solvent (20 μL min^{-1}) of 0.05% TFA in water:acetonitrile (95:5, v/v). The peptides were concentrated and desalted on a C_{18} PepMap™ Nano-Precolumn™ (5 mm × 0.3 mm i.d., 5 μm particle size; LC Packings, Amsterdam, The Netherlands) for 3 min, and eluted from the Nano-Precolumn™ and partially resolved on a C_{18} PepMap™ capillary column (15 cm × 75 μm i.d., 3 μm particle size; LC Packings), using a gradient flow of 200–300 nL min^{-1}. Three different solvent compositions and two different solvent gradients were examined (Table 1). When a gradient completed, the column was rinsed with 95% solvent B and equilibrated at 5% solvent B before injection of the next sample.

2.3. Enzymatic digestion of rMoAChE

rMoAChE (5.5 μM) was prepared in buffer (10 mM Tris–HCl, 100 mM NaCl, 40 mM $MgCl_2$, 0.02% NaN_3, pH 8) or 1.4 μM in the same buffer with 50% glycerol and stored at −20 °C prior to use. For rMoAChE stored in 50% glycerol solution, 16 μL of the protein was mixed with 84 μL of 25 mM NH_4HCO_3 and separated from glycol polymers in a H_2O-rinsed microcon YM-10™ Eppendorf filter by centrifugation at 14,000 × *g* for 15 min. The microcon filter was then inverted in an Eppendorf tube and centrifuged at 1000 × *g* for 3 min to retrieve the protein. For the rMoAChE stored without glycerol, 4 μL of the protein was used without filtering. Prior to digestion, rMoAChE was either denatured in 8 M urea in NH_4HCO_3 buffer for 1 h at 37 °C or buffer alone was added. Samples were diluted to afford a 2 M urea concentration whereupon chymotrypsin in 25 mM NH_4HCO_3 was added to produce the desired rMoAChE:chymotrypsin ratios. The solutions were incubated for 24 ± 4 h at 25 °C or at 37 °C. Acetonitrile, H_2O, and 10% trifluoroacetic acid (TFA) were added to the digest to produce final concentrations of 5%, 95% (H_2O + buffer), and 0.05%, respectively, and stored at −20 °C prior to chromatography and MS analysis.

2.4. Inhibition of rMoAChE by OPs

Stock solutions of methyl paraoxon (40 μM in acetone) and ethyl paraoxon (8 μM in acetone) were prepared and stored at 4 °C. rMoAChE (8 μM) in buffer was incubated with an equal volume of the OP in 25 mM NH_4HCO_3 for 20 min (methyl paraoxon) or 2 h (ethyl paraoxon), which resulted in >90% inactivation of rMoAChE.

2.5. MS analysis and identification of rMoAChE peptides

Nanoelectrospray (+) mass spectra were acquired throughout the chromatographic analyses on a QTOF-I (Waters Corp) MS, using the nanosprayer supplied by the manufacturer, and CID spectra were acquired in a data-dependent fashion on the most abundant ions having mass to charge ratios (*m*/*z*) from 400 to 1500. The capillary and sample cone were operated at 3500 V and 20 V, respectively. These voltages were determined to be optimum by observing the intensity of the doubly protonated $AChE_{14S}$ ion at *m*/*z* 650.3, relative to doubly protonated GFP (a very hydrophilic peptide, with GRAVY score of −1.107) ion at capillary voltages between 2500 V and 4000 V and sample cone voltages between 10 V and 60 V. The collision cell was pressurized with 1.5 psi ultra-pure Ar (99.999%), and collision voltages were dependent on the *m*/*z* ratio and the charge state of the parent ion. A calibration file was derived for the fragment ions. The CID of GFP was generated every fifth sample, processed using Mass Measure (Waters Corp.) and used to calibrate the five proceeding sample data files. Processed data defining peptide fragments were submitted to MASCOT (http://www.matrixscience.com/) for MSMS search or to Proteinlynx Global Server 1.0 (Waters Corp.) and searched against all mammalian entries in the NCBI non-redundant database. The protein/peptide search criteria were set with a mass accuracy of 40 ppm, mw 50–80 kDa, allowance for two missed cleavages, and variable oxidation of methionine. Chymotrypsin preferentially cleaves at phenylalanine (F), tyrosine (Y), tryptophan (W), and leucine (L). Manual identification of native and modified $AChE_{14S}$ was accomplished by generating extracted ion chromatograms (EIC) at the calculated *m*/*z* ± 0.1.

2.6. Statistical analyses

All statistical analyses were performed on SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). One-way analysis of variance was used to determine the differences between three or more data sets. When differences were found between means, Tukey's Honestly Significant Difference (hsd) was used as a *post-hoc* test to differentiate the means.

3. Results and discussion

3.1. Analysis of proteolytic peptides

The grand average hydropathicity score (GRAVY; 4.6 to −4.6) provides a relative measure of peptide hydrophobicity/hydrophilicity where scores larger in magnitude than ±1

are representative of high hydrophobicity or hydrophilicity. A negative score indicates a hydrophilic peptide and a positive score indicates a hydrophobic peptide. Trypsin digestion of rMoAChE results in LALQWVQENIAAFGGDPMSVTLFGESAGAASVGMHILSLPSR, the active-site-containing peptide with a monoisotopic mass of 4327.17 Da and a GRAVY score of 0.507. Chymotrypsin digestion of rMoAChE forms the active-site serine-containing peptide GESAGAASVGMHIL, with a monoisotopic mass of 1298.62 Da and a GRAVY score of 0.736 [17]. We hypothesized that our initial failure to identify the active-site peptides from tryptic and chymotryptic digests of rMoAChE was due to their hydrophobicity. Hydrophobic peptides can be difficult to analyze because they dissolve poorly in typical aqueous HPLC mobile phases [18] and are retained on reversed-phase columns to produce delayed elution times, band broadening, and signal reduction. Further, hydrophobic peptides do not compete well with more hydrophilic peptides for electrospray ionization [19]. Other investigators have reported the failure to identify the native, active-site serine peptide from AChE even when the OP-AChE conjugate has been identified [20].

Because we believed that the large tryptic peptide would pose greater difficulty dissolving in aqueous solution, eluting from the capillary chromatography column, and fragmenting during CID analysis, we pursued identification of the chymotryptic active-site peptide. Two synthetic peptides representing portions of rMoAChE were used to test instrumental conditions in this study. The decapeptide TLFGESAGAA ($AChE_{10S}$), a key sequence containing the active-site serine [14] and GESAGAASVGMHIL ($AChE_{14S}$), a chymotryptic, active-site-containing peptide were analyzed as described.

3.2. Chromatographic analysis of peptides

Modification of the organic phase has been shown to improve the MS signal for hydrophobic proteins and peptides [18,21], for example, addition of 5% hexafluoro-2-propanol (HFIP) prevents hydrophobic proteins from precipitating [22]. Similarly, the ESI signal of hydrophobic compounds and peptides increased when neat HFIP [18] or dimethylsulfoxide (DMSO) [19] were used. Schaller et al. [21] noted that the hydrophobic subunits of the mannose transporter complex were lost when the gradient solvents employed mixtures containing TFA in acetonitrile, methanol or chloroform but were recovered when the gradient solvents were 0.1% TFA or neat formic acid. Collectively, these studies suggest that hydrophobic peptide analysis is aided by HFIP and DMSO as a result of improved dissolution and elution. Therefore, in order to increase the probability of identifying the hydrophobic active-site peptide following chymotryptic digestion, rMoAChE samples were speed-vacuumed to dryness followed by dissolution in 5–50% DMSO or HFIP before injection on the capillary LC. Unfortunately, these experiments did not improve the signal:noise ratio of $AChE_{10S}$ when it was injected with chymotryptic rMoAChE digests, nor was identification of the active-site peptide possible (data not shown).

Retention on the C_{18} capillary column was a possible cause of peptide loss and poor signal:noise ratio, therefore the solvent system was modified. 2-Propanol [23] and dimethylsulfoxide (DMSO) [19] have been shown to be more effective than methanol or acetonitrile in eluting hydrophobic peptides. These findings led to the following changes to solvent B: (I) 0.2% TFA in acetonitrile, (II) 0.2% TFA in 2-propanol:acetonitrile (10:90, v/v), or (III) 0.2% TFA in DMSO:acetonitrile (10:90, v/v). The signal:noise ratios using these modified conditions to examine $AChE_{10S}$ showed that solvent II gave the highest MS signal:noise ratio when the peptide was injected alone or co-injected with rMoAChE digest (analysis of variance and Tukey's hsd, $\alpha = 0.10$). The ESI signal might also be enhanced in the presence of 2-propanol, and the observed effect might be due to a combination of enhanced chromatographic efficiency and ESI signal.

Using 10% 2-propanol in capillary LC solvent B with a gradient slope of 3% min^{-1} (5–50% solvent B over 12 min) led to detection of the desired doubly-charged ion at m/z 650.3 from a chymotryptic digest of rMoAChE (Fig. 2a and b). However, the base peak in the m/z 650.3 EIC had a poor signal:noise ratio of 3:1 and was not chosen for CID analysis due to a large number of co-eluting ions that were greater in intensity. A decrease in the gradient slope to 0.6% min^{-1} (5–40% solvent B over 60 min) yielded better chromatographic resolution. The base peak in this m/z 650.3 EIC trace showed a signal:noise ratio of 49:1 and m/z 650.3 was the base ion and was chosen for CID analysis (Fig. 2c and d). When the 3% gradient was used, 25 ions with intensity >10% of the base ion were seen in the mass spectrum (Fig. 2b). When gradient 2 was used, only five ions with intensity >10% of the base ion were seen in the mass spectrum (Fig. 2d). Nine of the co-eluting ions using the 3% gradient (Fig. 2b) were the correct mass to be identified as AChE peptides by a peptide mass fingerprint (Table 2). However, only three of the ions at this retention time were chosen for CID analysis and identified as AChE peptides by MASCOT. Most of these co-eluting ions were resolved when gradient 2 was used, and seven of the nine ions were chosen for CID analysis and identified as AChE ions by MASCOT (Table 2). Overall, decreasing the slope of the LC gradient increased the number of AChE peptides identified from 25 to 36, and increased protein coverage of rMoAChE from 42% to 63%.

Table 2
Peptides co-eluting with $AChE_{14S}$ using gradient 1, and subsequent resolution using gradient 2

m/z	Peptide	Relative retention time[a]	
		Gradient 1	Gradient 2
725.9^{2+}	ALPGSREAPGNVGLL	1.13[b]	1.28[b]
453.8^{2+}	SLPSRSLF	1.13	1.49[b]
450.2^{2+}	LAQVEGAVL	1.13	1.65[b]
762.4^{2+}	ARTGDPNDPRDSKSPQWPPY	1.13	1.65
650.3^{2+}	GESAGAASVGMHIL	1.13	1.71[b]
776.4^{2+}	IYGGGFY	1.13[b]	1.74
500.8^{2+}	NRFLPKLL	1.13[b]	1.76[b]
535.3^{2+}	INTGDFQDL	1.13	1.76[b]
535.3^{2+}	HVLPQESIF	1.13	1.86[b]

[a] Retention time is relative to retention of GFP which was co-injected with rMoAChE digest.
[b] Indicates CID analysis and positive identification as AChE peptide by MASCOT.

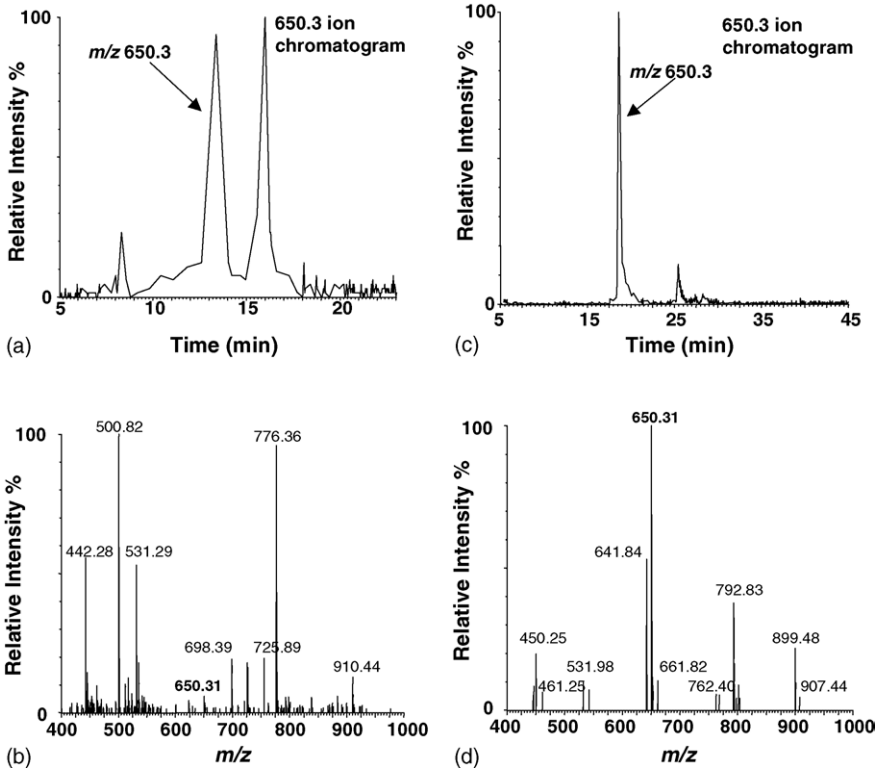

Fig. 2. (a) m/z 650.3 ± 0.1 extracted-ion chromatogram resulting from the analysis of a chymotryptic digest of rMoAChE using gradient 1 (Table 1); (b) mass spectrum under the m/z 650.3 peak in a; (c) m/z 650.3 ± 0.1 extracted-ion chromatogram resulting from the analysis of a chymotryptic digest of rMoAChE using gradient 2 (Table 1); (d) mass spectrum under the m/z 650.3 peak in c.

The mass spectrum resulting from CID analysis of m/z 650.3 in Fig. 2d revealed a nearly complete b- and y-ion series for the expected rMoAChE active-site peptide with b_3 through b_7 also forming dehydroalanine ions (indicated by b_x^*) through loss of H_2O (Fig. 3).

3.3. Proteolytic digestion of rMoAChE

Chymotryptic digestion of rMoAChE affords the putative active-site-containing peptide $AChE_{14S}$. However, subtle variations in sample preparation led to inconsistent identification of this active-site peptide. Two experimental variables were addressed: (a) denaturing rMoAChE prior to enzymatic digestion to permit more complete cleavage, and (b) controlling the ratio of chymotrypsin to rMoAChE to ensure fully competent cleavage unencumbered by excess chymotrypsin, autocatalytic peptide, and non-specific peptide interference. Previous studies have denatured AChE before protease digestion using heat or chemical reagents [10,24,25] or used no denaturation [11,12,26] prior to digestion by trypsin. In these reports, AChE was not

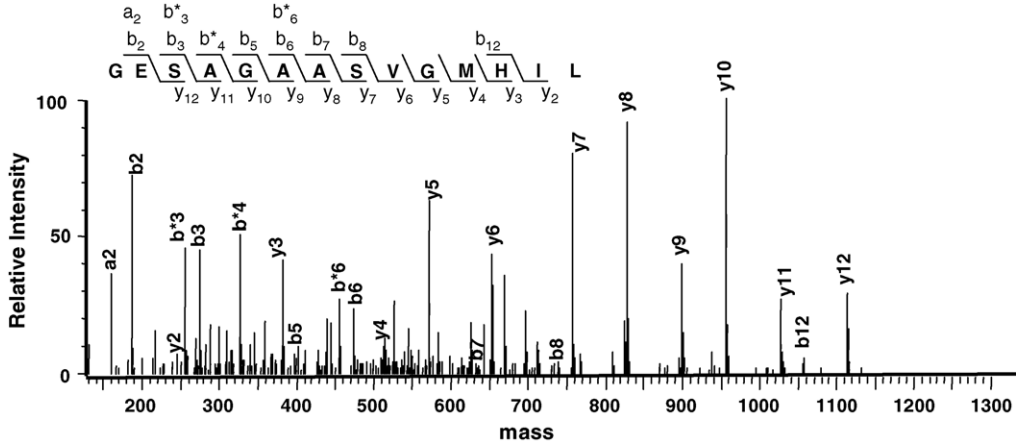

Fig. 3. De-isotoped mass spectrum resulting from CID analysis on the m/z 650.3 ion in Fig. 2d. Fragment ion nomenclature from Biemann [31]. (*) indicates dehydroalanine (loss of H_2O).

reduced (disulfide cleavage) or alkylated at cysteine prior to digestion. We compared chymotryptic digests of rMoAChE that was denatured using heat, 8 M urea [27], or using no denaturation and found that pretreatment had no effect and in some cases reduced the net protein coverage map compared to control. Although re-folding of AChE in 2 M urea after dilution and addition of chymotrypsin is a possible concern, we did not reduce or alkylate because of the possibility that phosphorylated residues might be lost during chemical treatment. Since various denaturation conditions failed to produce an increase in protein coverage, the ratio of protease to rMoAChE was examined.

In previously published studies, chymotryptic digestions used protein:chymotrypsin molar ratios ranging from 2:1 to 2000:1 in varying reaction times and reaction temperatures [28–30]. In our experiments, rMoAChE:chymotrypsin molar ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 were examined at room temperature and 37 °C to determine which conditions produced the maximum number of chymotryptic peptides with the least number of non-specific cleavages. The efficiency of chymotrypsin digestion was studied by MS and CID analysis, using an Automod™ query on Proteinlynx Global Server 2.0 to identify the number of chymotrypsin-specific cleavages. In this analysis, the predicted chymotryptic peptides were defined as cleavages at the C-terminal to F, Y, W, and L, and non-specific peptides resulted from cleavage at the C-terminal to any other amino acid. Presumably, at low rMoAChE:chymotrypsin ratios (high chymotrypsin amount) and higher temperatures, chymotrypsin would cleave more competently at the C-termini of phenylalanine, tyrosine, tryptophan, and leucine, but the number of non-specific cleavages would also increase. The digestion results are shown in Fig. 4a (25 °C) and Fig. 4b (37 °C).

Although variations in chromatography made it difficult to find statistically significant differences in the numbers of peptides identified, the trends were the same in duplicate experiments, namely, at 25 °C the number of rMoAChE peptides identified increased as the rMoAChE:chymotrypsin ratio decreased to 1:2. At and beyond a ratio of 1:2, the number of non-specific peptides increased. When the digestion temperature was increased from 25 °C to 37 °C, the overall number of chymotryptic peptides increased relative to the amount found at 25 °C. However, the number of non-specific peptides also increased as the rMoAChE:chymotrypsin ratio decreased. The digestion temperature and enzyme ratio were crucial to identifying the active-site peptide, which was only identified consistently at rMoAChE:chymotrypsin ratios of 5:1 to 2:1 for digestion at 37 °C and 1:5 for digestion at 25 °C. Experiments in which digestion at room temperature was followed by western blot analysis using anti-mAChE, which will only bind the intact rAChE protein, correlated well with results from MS analyses (data not shown).

3.4. Identification and sequencing of OP-adducted peptides derived from OP-AChE conjugates (inactivated rMoAChE)

Methyl paraoxon and ethyl paraoxon were reacted with rMoAChE at the ratios required for ≥90% inhibition, digested

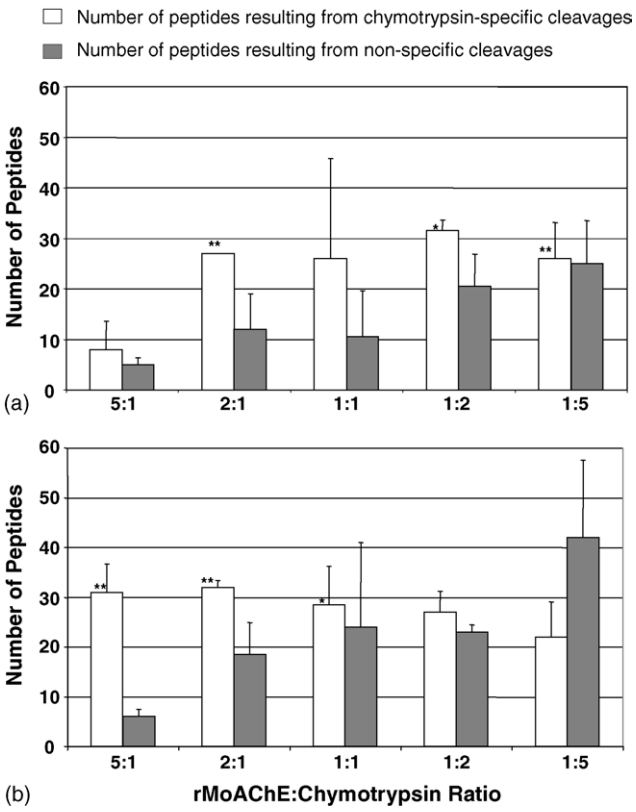

Fig. 4. The number of peptides resulting from chymotryptic cleavages at both ends (□) and the number of peptides resulting from non-specific cleavages at one or both ends (■), as identified using the Automod™ analysis on Proteinlynx Global Server 2.0: (a) reaction carried out for 24 h at 25 °C (no denaturation) and (b) reaction carried out for 24 h at 37° (no denaturation). Data points are the result of duplicate analyses; error bars = 1σ. (*) indicates identification of $AChE_{14S}$ in one of two samples analyzed. (**) indicates identification of $AChE_{14S}$ in two of two samples analyzed.

with chymotrypsin, and analyzed by CapLC/QTOF using optimized instrumental conditions. We expected methyl paraoxon inactivation of rMoAChE followed by chymotryptic digestion to result in an active-site peptide of mw 1406.62 with the expected ion at 704.32^{2+} (Fig. 1). Under optimized conditions, the base peak in the *m*/*z* 704.3 EIC from rMoAChE inactivated with methyl paraoxon had a signal:noise ratio of 67:1 (Fig. 5a) (other peaks in the chromatogram were from ions of incorrect *m*/*z* or incorrect charge). The base ion in the mass spectrum under the peak was 704.34^{2+} (Fig. 5b). In contrast, the base peak in the *m*/*z* 650.3 EIC (not shown) had a signal:noise ratio of 33:1, and the 650.3^{2+} ion in this peak was too small for CID analysis, indicating that most of the rMoAChE was indeed inactivated. CID analysis of *m*/*z* 704.34 revealed a complete y-ion series from y_5 to y_{11}, indicating no modification of the serine at position 8 in the peptide (S_8). Previous MS analysis of $AChE_{10S}$ conjugated at serine to $–P(O)(OH)(OCH_3)_2$ or $–P(O)(OH)(OC_2H_5)_2$ indicate that y or b fragments containing native serine residues occur only from fragmentation of the native peptide, i.e., during fragmentation the OP-conjugated serine cannot revert to its native state (unpublished data, R.S. Spaulding). We expected that the modified peptide would fragment to form dehydroalanine by loss of $P(O)(OH)(OCH_3)_2$. However, the highest inten-

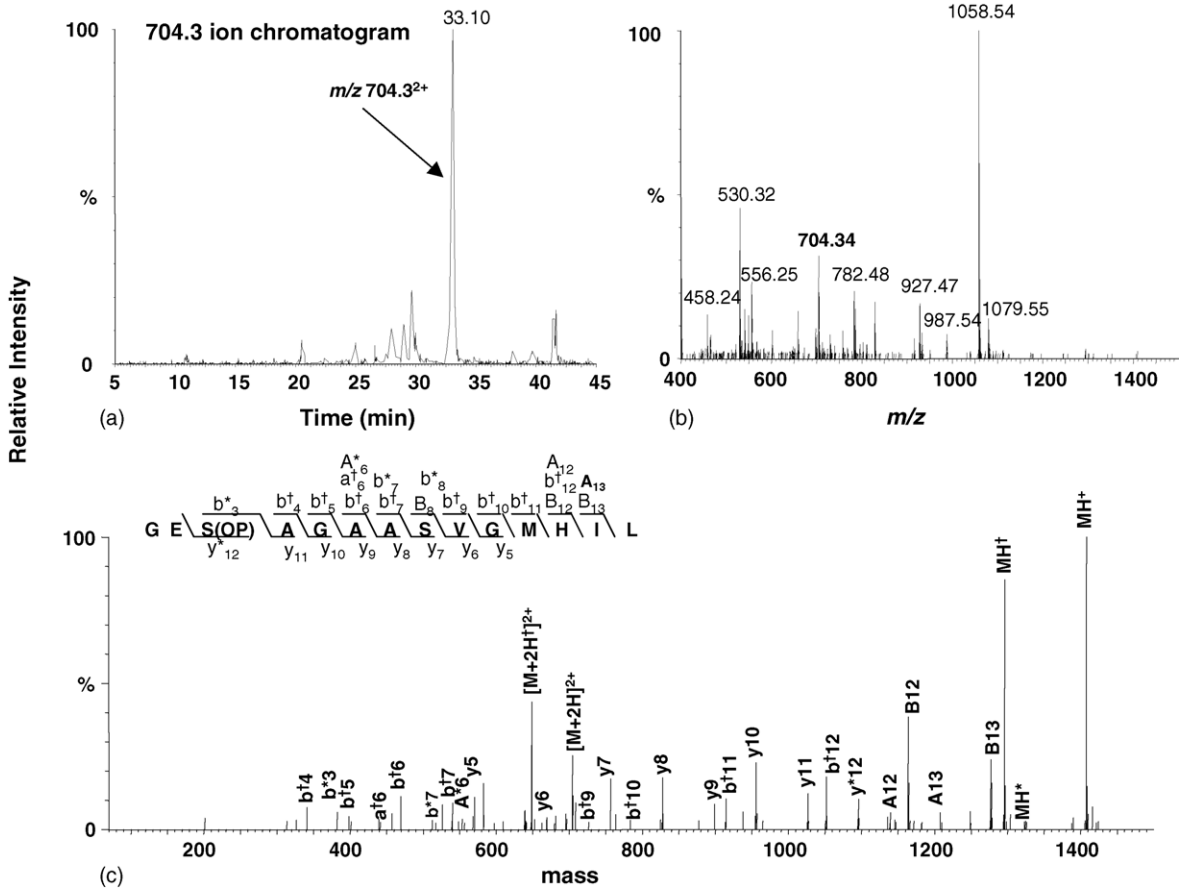

Fig. 5. (a) *m/z* 704.3 extracted-ion chromatogram resulting from the analysis of a chymotryptic digest of methyl paraoxon-treated rMoAChE using optimized instrumental conditions (Table 2); (b) mass spectrum under the *m/z* 704.3 peak in (a); and (c) de-isotoped mass spectrum resulting from CID on the *m/z* 704.3 ion in b. Fragment ion nomenclature from Biemann [31]. (*) indicates dehydroalanine (loss of H_2O or $P(O)(OH)(OCH_3)_2$); (†) indicates loss of $P(O)(OH)_2(OCH_3)$; a_x, b_x and y_x indicate peptide fragments with native serine residues; A_x, B_x and Y_x indicate peptide fragments with $P(O)(OCH_3)_2$ on one serine residue.

sity peaks in this spectrum were $[M+H]^+$ at *m/z* 1407.69 and $[M+H–(P(O)(OH)_2(OCH_3)]^+$ at *m/z* 1295.73^+ (indicated by $MH^\dagger$ in Fig. 5c). The methyl paraoxon-modified peptide (Fig. 1; R = Me) appears not to have formed dehydroalanine, but to have undergone a rearrangement that left one of the methyl groups from the OP on the peptide when the phosphate group was lost. This rearranged peptide then fragmented to a complete $b^\dagger$-ion series from b_4 through b_{12}, with no b (serine in native state) or B (serine with intact OP group) ions present, in contrast to the native peptide whose b ions fragmented both intact and in the dehydroalanine form (Fig. 3). This fragmentation pattern indicates that the OP modification was at the serine at position 3 in the peptide (S_3). B_{12}, B_{13}, A_{12}, and A_{13} were the only ions seen with the intact methyl-paraoxon modification.

We expected ethyl paraoxon inactivation of rMoAChE (Fig. 1; R = Et) followed by chymotryptic digestion to result in an active-site peptide with mw 1423.66 with an expected ion of 718.34^{2+}. The base peak in the *m/z* 718.3 EIC from rMoAChE inactivated with ethyl paraoxon had a signal:noise ratio of 167:1 (Fig. 6a) (other peaks in the chromatogram were from ions of incorrect *m/z* or incorrect charge). The base ion in the mass spectrum under the peak was 718.34^{2+} (Fig. 6b). In contrast, the base peak in the *m/z* 650.3 EIC had a signal:noise ratio of 16:1 (not shown), and the 650.3^{2+} ion in the peak was too small for CID analysis, indicating that most of the rMoAChE was indeed inactivated. CID analysis of *m/z* 718.34 revealed a nearly complete y-ion series up to y_{10} (serine in native state), where Y_{12} and Y_{13} would include the modified serine base if the OP modification occurred at S_3 (Fig. 6c). Although Y_{12} and Y_{13} were not present, the presence of y_7 through y_{10} indicates that S_8 was not modified. Unlike the methyl paraoxon-modified peptide, this peptide formed the expected dehydroalanine fragment ions without any rearrangement. High intensity dehydroalanine ions from MH^+ and y_{12} (indicated by MH^* and y^*_{12}), which were not seen in CID analysis of the native peptide (Fig. 3), were observed, supporting modification at S_3. Only 2 intact b ions were seen: b_2 (serine in native state), which occurs prior to S_3, and B_8, which is the only fragment observed with an intact OP-modification. However, dehydroalanine peaks were observed for b_4 through b_{13} (indicated by b^*_x). This is in contrast to the CID spectrum from the native active-site peptide (Fig. 3) in which both b_x and b^*_x ions were observed, and again supports OP modification at S_3.

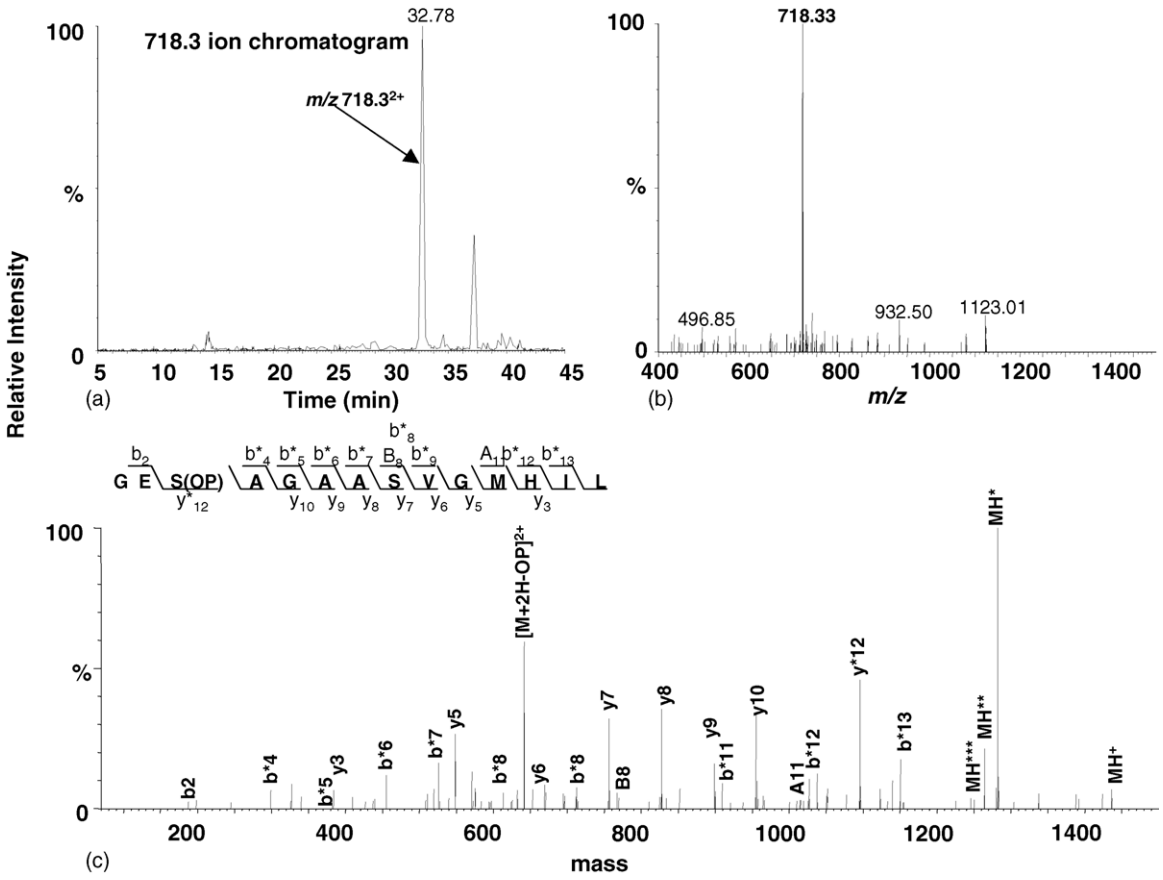

Fig. 6. (a) *m*/*z* 718.3 extracted-ion chromatogram resulting from the analysis of a chymotryptic digest of ethyl paraoxon-treated rMoAChE; (b) mass spectrum under the *m*/*z* 718.3 peak in a; and (c) de-isotoped mass spectrum resulting from CID on the *m*/*z* 718.3 ion in d. Fragment ion nomenclature from Biemann [31]. (*) indicates dehydroalanine (loss of H_2O or $P(O)(OH)(OC_2H_5)_2$); (**) indicates loss of $P(O)(OH)(OC_2H_5)_2$ and H_2O; (***) indicates loss of $P(O)(OH)(OC_2H_5)_2$ and two H_2O; a_x, b_x and y_x indicate peptide fragments with native serine residues; A_x, B_x and Y_x indicate peptide fragments with $P(O)(OC_2H_5)_2$ on one serine residue.

4. Conclusion

Chymotrypsin digestion of rMoAChE produced a peptide containing the active-site serine residue that was both lost during chromatography and competed poorly with the more hydrophilic peptides during ionization. Addition of 2-propanol to the capillary LC gradient solvent improved the signal of $AChE_{10S}$ when co-injected with a chymotryptic digest of rMoAChE, suggesting that either ionization and/or elution of the peptide was improved. Changing the linear solvent gradient from 3% min^{-1} to 0.6% min^{-1} solvent B (0.2% formic acid in 2-propanol:acetonitrile, 10:90, v/v) aided the identification and CID analysis of the active-site peptide and improved the protein coverage map of rMoAChE from 42% to 63%. Even with optimized parameters, variations in the ability to identify the chymotryptic rMoAChE active-site peptide were observed. This was overcome by rigorous examination of the digestion conditions and the molar ratio of proteolytic enzyme. In contrast to prior reports, AChE did not require chemical or physical denaturation prior to digestion with chymotrypsin [10,24,25]. Moreover, a 2:1 molar ratio of rMoAChE:chymotrypsin (24 h at 37 °C) produced the maximum active-site peptide score and number of ions identifiable as chymotryptic peptides. The method was successfully used to sequence native and phosphylated active-site peptides from untreated, methyl paraoxon-inactivated, and ethyl paraoxon-inactivated rMoAChE. Moreover, CID analysis allowed sequencing of the active-site peptide and identification of the exact amino acid that had been modified by the OP. The study established the precise serine that is phosphylated by reactive organophosphates and therefore makes possible the identification of native and modified AChE purified proteins.

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