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PHOS-Select Iron Affinity beads enrich peptides for detection of organophosphorus adducts on albumin

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Abstract

Albumin is covalently modified by organophosphorus toxicants (OP) on tyrosine 411, but less than 1% of albumin is modified in humans by lethal OP doses that inhibit 95% of plasma butyrylcholinesterase. A method that enriches OP-modified albumin peptides could aid analysis of low dose exposures. Soman or chlorpyrifos oxon treated human plasma was digested with pepsin. Albumin peptides were enriched by binding to Fe³⁺ beads at pH 11 and eluted with pH 2.6 buffer. Similarly, mouse and guinea pig albumin modified by chlorpyrifos oxon were digested with pepsin and enriched by binding to Fe³⁺ beads. Peptides were identified by MALDI-TOF/TOF mass spectrometry. PHOS-select Iron Affinity beads specifically enriched albumin peptides VRY₄₁₁TKKVPQVST and LVRY₄₁₁TKKVPQVST in a pepsin digest of human plasma. The unmodified as well as OP-modified peptides bound to the beads. The binding capacity of 500 μ l beads was the pepsin digest of 2.1 μ L human plasma. The limit of detection was 0.2% of OP-modified albumin peptide in 0.43 μ L plasma. Enrichment of OP-modified albumin peptides by binding to Fe³⁺ beads is a method with potential application to diagnosis of OP pesticide and nerve agent exposure in humans, mice, and guinea pigs.

Keywords

soaman; chlorpyrifos oxon; mass spectrometry; mouse; human; guinea pig; pepsin

Introduction

The butyrylcholinesterase enzyme in human plasma is the most sensitive biomarker of exposure to organophosphorus toxicants (OP). Mass spectrometry methods to measure OP

Supporting Information Available

Experiments on the isolation and enrichment of tyrosine-41 related peptides from mouse and guinea by PHOS-Select Iron Affinity chromatography are presented in the Supporting Information. Structures for organophosphates known to form adducts on tyrosine 411 of human albumin are also presented in the Supporting Information as well as a study on the importance of lysine and tyrosine in the affinity of the tyrosine 411 containing peptide for Fe³⁺. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

adducts on butyrylcholinesterase have identified exposure in humans.^{1, 2} Acetylcholinesterase in red blood cells and albumin in plasma are also covalently modified by organophosphorus pesticides and nerve agents. *In vitro* studies have shown that human albumin is covalently modified on tyrosine 411 by chlorpyrifos, chlorpyrifos oxon, dichlorvos, paraoxon, profenofos, diisopropyl fluorophosphate, sarin, soman, cyclosarin, tabun, VX, Chinese VX, Russian VX, FP-biotin, and cresyl saligenin phosphate.³⁻⁸ Structures for these organophosphates can be found in the Supporting Information, Figure S6. Guinea pigs and nonhuman primates treated with nerve agents have nerve agent-modified albumin in blood.^{9, 10} Mice treated with chlorpyrifos oxon, and rats treated with paraoxon have diethoxyphosphate-modified albumin in blood.^{11, 12} Three studies have provided mass spectrometry evidence for the presence of OP adducts on albumin in human subjects who poisoned themselves with dichlorvos or chlorpyrifos.¹³⁻¹⁵ Albumin is much less reactive with OP than butyrylcholinesterase because of the poor nucleophilicity of Tyr 411 compared to the active site serine in butyrylcholinesterase. A lethal dose of OP inhibits 95% of human plasma butyrylcholinesterase, but modifies less than 1% of albumin. To date mass spectrometry has detected adducts on albumin only in humans and animals exposed to high doses of OP.

Low level exposures that modify less than 1% albumin, require methods that enrich OP-modified albumin. Antibodies have been developed for this application but have not yet been tested with human exposure cases.¹⁶⁻¹⁸ A mass spectrometry method that detects OP-tyrosine in pronase-treated plasma samples has a limit of detection of 0.01 ng/mL.^{9, 19} This approach has been successfully applied to cases of human exposure to chlorpyrifos.¹³ A unique strategy for enrichment of tyrosine 411 containing albumin peptides has been developed by Russian scientists. Babakov et al. (personal communication) found that OP-labeled albumin as well as unlabeled tyrosine 411 containing peptides bind to Fe³⁺ affinity beads at pH 11 and elute at pH 2.6. This method is the reverse of that used to bind phosphopeptides to Fe³⁺ beads, where the protocol is to bind at low pH and elute at high pH. In this report we confirm that human albumin peptic-peptides VRY₄₁₁TKKVPQVST and LVRY₄₁₁TKKVPQVST are selectively enriched from a plasma digest by binding to Fe³⁺ beads at pH 11 and elution at pH 2.6. The unmodified as well as the OP-modified peptides bind to Fe³⁺ affinity beads. The strategy works not only with OP-modified pure human albumin, but also with albumin in human plasma, and with OP-modified mouse and guinea pig albumin. The limit of detection with this enrichment strategy is 0.2% (labeled albumin to unlabeled) OP-labeled albumin from 0.43 μ l human plasma. This method for enriching the OP-labeled, tyrosine 411-containing albumin peptide has potential application for analysis of OP exposure *in vivo*.

Materials and methods

Racemic soman was from DGA Maîtrise NRBC (Vert-le-Petit, France) and from RIHOPHE, Russia. Soman is a schedule I chemical as defined in the Chemical Weapons Convention. Samples treated with soman were prepared by qualified personnel in the Institut de Recherche Biomedicale des Armées-CRSSA in La Tronche, France and in RIHOPHE, Russia. The stock solution of soman was in isopropanol. *O*, *O*-diethyl *O*-3, 5, 6-trichloro-2-pyridyl phosphate (chlorpyrifos oxon or CPO) (ChemService, cat# Met-674B; West Chester, PA, USA) was dissolved in ethanol and stored at -20°C. A saturated solution (10 mg/mL) of α -cyano-4-hydroxycinnamic acid (CHCA) matrix (Applied Biosystems, Foster City, CA, USA) was prepared in 50% acetonitrile, 0.1% trifluoroacetic acid (v/v). A 0.4 M ammonium hydroxide (Fisher Scientific, Pittsburgh, PA, USA, a member of the Thermo Scientific group, cat# A669) solution in 30% acetonitrile (v/v) pH 11 was prepared immediately before use. Stored 0.4 M ammonium hydroxide solutions were not used because the pH dropped below 11. A 250 mM acetic acid (Fisher Scientific, cat# A38) solution in 30% acetonitrile

(v/v) had a pH value of 3.0. Other reagents and their sources were porcine pepsin (Sigma, cat# P-6887), human albumin free of fatty acids (Fluka a member of the Sigma-Aldrich Co, cat# 05418), mouse albumin (Innovative Research, Novi, MI, USA), guinea pig albumin (Equitech-Bio Inc., cat# GPSA62), and human plasma (University of Nebraska Hospital Blood Bank).

PHOS-Select™ Iron Affinity Gel (Sigma, cat# P9740) is an iron <Fe (III)> chelate matrix. The resin is supplied as a 50% suspension (v/v) in 50% glycerol (v/v) buffered at pH 5 and stored at -20°C. PHOS-Select Iron Affinity Gel beads were completely suspended and immediately loaded into special tips (TopTip Empty 1–10 µL, part number TT1EMT.96, Glygen Corp., Columbia, MD, USA) to make mini-columns.

Treatment of pure albumin or human plasma with CPO

A 1 mL aliquot of pure human, mouse, or guinea pig albumin (1 mg/mL) in 10 mM NH_4HCO_3 pH 8 was treated with 5 µL of CPO (50 mM stock in ethanol) and incubated at 37°C for 3 days. 70% of human albumin, 87% of mouse albumin and 88% of guinea pig albumin was labeled on Tyr 411. Human plasma (collected in acid-citrate-dextrose) was diluted 40-fold with 10 mM NH_4HCO_3 pH 8 to yield a plasma albumin concentration of approximately 1 mg/mL. A 1 mL aliquot of diluted human plasma was treated with 10 µL of CPO (0.3 M stock in ethanol) and incubated at 37°C for 2 days. About 70% albumin in human plasma was labeled by CPO. When plasma was not diluted before addition of OP, the percent albumin modified was much lower.

Estimation of percent OP-modified albumin

Percent labeled albumin was estimated from peak areas of unlabeled and labeled peptides produced by digestion with pepsin and analyzed by MALDI-TOF mass spectrometry. The unlabeled and labeled peptides were in the same spot of the MALDI plate.

Treatment of pure human albumin with soman

Human albumin (1 mg/mL) in 20 mM Tris/HCl pH 8 was treated with 0.15 mM soman at room temperature for 7 days. MALDI-TOF analysis of pepsin digested albumin indicated that 27% of the albumin was covalently modified on Tyr 411. When human plasma is treated for 7 days at room temperature with 0.15 mM racemic soman, plasma butyrylcholinesterase is inhibited 95%.

Pepsin digestion of albumin or human plasma

The pH of 100 µL albumin (1 mg/mL) or of 100 µL diluted human plasma (1 mg/mL, equivalent to 2.5 µL of plasma) with no treatment or treated with CPO (0.3 M for 2 days at 37°C) or soman (0.15 mM for 7 days at room temperature) was adjusted to pH 2 with 1 µL of 25% trifluoroacetic acid (v/v). A fresh solution of 2 mg/mL pepsin in 5% formic acid (v/v) was prepared just before use. The albumin or human plasma was digested with 15 µL pepsin (30 µg) at 37°C for 2 h. Final concentrations of trifluoroacetic and formic acids were 0.25% and 0.45% (v/v), respectively. A 1 µL aliquot of each digest was analyzed by MALDI-TOF mass spectrometry for comparison with samples enriched on PHOS-Select Iron Affinity Gel.

Sample pH adjusted to pH 11—Samples were prepared for enrichment of OP-labeled albumin peptides by adjusting the pH of the peptic digests to pH 11. For human albumin and human plasma, 10 µL digest was mixed with 60 µL of 0.4 M ammonium hydroxide pH 11 in 30% acetonitrile (v/v). For mouse albumin and guinea pig albumin, 2 µL digest was mixed

with 68 μL of 0.4 M ammonium hydroxide pH 11 in 30% acetonitrile (v/v). The pH was checked with pH paper and adjusted to pH 11 with 1 M NaOH when necessary.

Limit of detection using 60 μL of PHOS-Select Iron Affinity Gel—Diluted human plasma (40-fold diluted to yield 1 mg/mL albumin) treated with CPO to label 70% of Tyr 411 residues, was used for determination of the limit of detection. Excess CPO was removed by dialysis. The protein concentration of the dialyzed CPO-treated plasma was adjusted to match the untreated diluted control human plasma. Untreated human plasma (1 mg/mL) was mixed with dialyzed CPO-plasma to give 0.1%, 0.2%, 1.4%, 2%, 2.5%, 3.5% and 7% CPO-albumin in plasma (in a final volume of 100 μL). The pH of 100 μL diluted human plasma (1 mg/mL) with different CPO labeling percentages was adjusted to pH 2 with 1 μL of 25% trifluoroacetic acid (v/v). Samples were digested with 15 μL pepsin (30 μg) at 37°C for 2 h. A 10 μL aliquot of each digest was mixed with 60 μL of 0.4 M ammonium hydroxide pH 11 in 30% acetonitrile (v/v) and used for enrichment on 60 μL iron affinity beads (packed bed volume). The 10 μL aliquot of 1 mg/mL human plasma contains albumin equivalent to 0.25 μL of undiluted human plasma (the albumin concentration in plasma is 40 mg/mL).

Albumin peptides were enriched on PHOS-Select Iron Affinity Gel beads packed into microcolumns (60 μL bed volume). The microcolumns were prepared by pipetting a suspension of PHOS-Select Iron Affinity Gel beads into special tips (TopTip Empty 1–10 μL , part number TT1EMT.96, Glygen Corp., Columbia, MD, USA). The tips were placed into centrifuge adaptors that hold tips in 1.5 mL microfuge tubes. The centrifuge adaptors are sold with the empty TopTips. Tips were centrifuged for 10 min at 2000 rpm ($300 \times g$) in a microcentrifuge (Spectrafuge 24D from Labnet International Inc, Edison, NJ, USA). The packed bed height of the beads in the tips was 2 cm (about 60 μL).

Beads were washed with 75 μL of water, 3 times, and then with 75 μL of 0.4 M ammonium hydroxide pH 11 in 30% acetonitrile (v/v), before samples were loaded. Samples with a volume of 70–75 μL in pH 11 buffer were pipetted on top of the relatively dry bed of beads, where they were absorbed into the gel. Tips were incubated 30 min before they were centrifuged at 2000 rpm in the Spectrafuge 24D for 10 min. The eluted liquid was reapplied to the tips 3 more times, without an incubation period. After the samples had passed through the tips a final time, the tips were washed 3 times with 75 μL of 0.4 M ammonium hydroxide containing 30% acetonitrile (v/v) and 3 times with 75 μL of water to wash off unbound peptides. Bound peptides were released with two 50 μL applications of 250 mM acetic acid pH 3.0 in 30% acetonitrile (v/v). The released peptide solutions were dried by vacuum centrifugation and dissolved in 5 μL of 5% acetonitrile, 0.1% trifluoroacetic acid (v/v). One μL aliquots were spotted onto a 384-well target plate (cat#. 1016629, Applied Biosystems, Foster City, CA, USA), dried, and overlaid with 1 μL of CHCA matrix in preparation for analysis in the MALDI-TOF/TOF mass spectrometer.

Limit of detection using 500 μL of PHOS-Select Iron Affinity Gel—To increase the limit of detection from 2% to 0.2% of CPO-modified albumin, it was necessary to increase the volume of beads, thus allowing binding of a higher volume of digested plasma. Pepsin digests of diluted plasma samples were prepared as described above. The entire 100 μL digest was mixed with 600 μL of 0.4 M ammonium hydroxide pH 11 in 30% acetonitrile (v/v) and enriched on 500 μL iron affinity beads. The 100 μL aliquot is equal to 2.2 μL of undiluted human plasma (40 mg albumin/mL).

The 500 μL PHOS-Select Iron Affinity beads were packed into a 1 mL microcolumn that fit inside a standard 1.5 mL microfuge tube. The beads were washed by centrifugation using the protocol described above, with the difference that the wash volumes were increased to

500 μ L. Bound peptides were released with two 250 μ L applications of 250 mM acetic acid pH 3.0 in 30% acetonitrile (v/v).

Mass spectrometry

MALDI mass spectra were acquired on a MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems, Framingham, MA, USA). Mass spectra were taken in positive reflector mode with CHCA matrix. Each spectrum was the average of 3000 laser shots with the laser energy adjusted to 5000 V, delayed extraction set to 625 ns, low mass gate set to on, and low mass gate offset to zero. The identity of peaks observed in MS spectra was confirmed by MS/MS analysis. MS/MS spectra were acquired in positive mode using post source decay with CID set to on, with air as the collision gas, gas pressure set to medium (2×10^{-6} Torr), precursor mass window set to relative 200, delayed extraction DE1 set to 260 ns and DE2 set to 36,518 ns, metastable suppression set to on, and timed ion selector set to on. Spectra were analyzed with Data Explorer Software. The mass spectrometer was calibrated against Calibration Mixture 5 in MS mode and against Angiotensin 1 in MS/MS mode, both standards being present on the Opti-TOF mass standards calibration insert (Applied Biosystems, cat# 4358092).

Results

PHOS-Select Iron Affinity Gel beads enrich the tyrosine 411 peptides from pure human albumin

The MS spectrum of pepsin digested pure human albumin is shown in Figure 1A. The masses at 1406 and 1519 m/z are for peptides VRY₄₁₁TKKVPQVST and LVRY₄₁₁TKKVPQVST. Both peptides include tyrosine 411. These two peptides bind to iron beads at pH 11 and are recovered by elution with 250 mM acetic acid in 30% acetonitrile pH 3 (v/v) as shown in Figure 1B. The intensity of the 1406 and 1519 m/z peaks increased after enrichment on PHOS-select Iron Affinity beads.

Figure 1B showed a remarkable selectivity of Fe³⁺ beads for enriching only tyrosine 411 containing peptides of human albumin. The possibility was tested that additional peptides might bind to Fe³⁺ beads if albumin disulfide bonds were reduced and alkylated, before albumin was digested with pepsin. However, no other albumin peptides were enriched.

The digestion conditions were very important for achieving the enrichment demonstrated in Figure 1B. Longer peptides, containing 15 or 16 residues (VRYTKKVPQVSTPTL and LVRYTKKVPQVSTPTL) rather than 12 or 13 (VRYTKKVPQVST and LVRYTKKVPQVST), were produced by digesting with pepsin dissolved in 1 mM HCl, but the longer peptides did not bind well to Fe³⁺ beads. The critical factor for determining the size of the peptide was the identity and concentration of the acid in the digestion mixture; the pepsin concentration was less important. Pepsin digestion yielded the shorter peptides when the digest contained 0.22% trifluoroacetic acid and 0.45% formic acid.

Treatment of pure human albumin with CPO added a mass of 136 Da from diethoxyphosphate to tyrosine 411, as indicated by the scheme in Figure 2A. The MS spectrum in Figure 3A for the digest before enrichment shows a peak at 1406 m/z for the unmodified peptide and small peaks at 1542 and 1655 m/z for peptides VRY₄₁₁TKKVPQVST and LVRY₄₁₁TKKVPQVST modified on tyrosine 411 by CPO. Figure 3B shows that following enrichment of the digest on iron affinity beads, the intensity of the unmodified as well as the CPO-modified peaks increased.

Treatment of pure human albumin with soman added 162 Da from O-pinacolyl methylphosphonate bound to tyrosine 411 as indicated by the scheme in Figure 2B. The MS

spectrum in Figure 4A for the digest before enrichment shows a peak at 1406 m/z for unmodified peptide VRY₄₁₁TKKVPQVST and a peak at 1568 m/z for the same peptide modified on tyrosine 411 by soman. The intensity of both the 1406 m/z and 1568 m/z peaks increased after enrichment (Figure 4B). The results in Figures 1 and 3 had led to the expectation that pepsin digestion would yield two tyrosine 411 peptides, one with a missed cleavage. The unmodified peptide with a missed cleavage at 1519 m/z is present in Figures 4A and 4B, but its soman-labeled partner (expected to be at 1681 m/z) is not detectable.

An important result to note in Figures 3 and 4 is that the OP adducts on tyrosine have not aged. That is, the parent ions have not lost an alkyl group; the adduct from CPO has two diethoxy groups on the phosphorus atom, while the adduct from soman includes the pinacolyl group.

The MS/MS spectrum in Figure 5A confirms the amino acid sequence of unmodified peptide VRY₄₁₁TKKVPQVST. The MS/MS spectrum in Figure 5B identifies tyrosine 411 of human albumin as the residue modified by CPO; consistent with this assignment are a) the mass at 512.2 m/z for fragment YTK minus ammonia, b) the a3 ion at 527.3 m/z, c) the b3 minus ammonia ion at 538.1 m/z, and d) the b4 to b10 ion series. All ions beginning with the b3 ion are 136 Da heavier than in the unmodified peptide, a result that identifies the 3rd amino acid from the N-terminal as the modified residue. The diethylphosphotyrosine immonium ion at 272.1 Da supports the conclusion that the modified residue is tyrosine. The MS/MS spectrum in Figure 5C identifies tyrosine 411 as the residue modified by soman. The evidence for this assignment includes the fact that the b3 ion and all ions in the b4–b10 series are heavier by 162 Da than in the unmodified peptide. The methylphosphotyrosine immonium ion at 214.1 m/z in Figure 5C further supports the conclusion that the modified residue is tyrosine. The laser used for fragmentation of the peptide in Figure 5C caused the pinacolyl portion of the soman adduct to break off to yield the mass at 1483.6 m/z, and the entire pinacolyl methyl phosphonate group to break off to yield the mass at 1405.9 m/z.

PHOS-Select Iron Affinity Gel beads enrich the tyrosine 411 peptides from human plasma

The human plasma proteome database (www.plasmaproteomedatabase.org) lists 9,372 proteins as of July 2013. The protein concentration in human plasma is about 60 mg/mL. The most abundant protein in human plasma is albumin at a concentration of 40 mg/mL. The high concentration of albumin relative to other proteins suggested that albumin peptides might be detectable using the strategy developed for pure human albumin in Figure 1. To test this hypothesis, human plasma digested with pepsin was analyzed before and after enrichment on iron affinity beads. The MS spectrum in Figure 6A for the digest before enrichment shows barely detectable peaks at 1406 and 1519 m/z for peptides VRY₄₁₁TKKVPQVST and LVRY₄₁₁TKKVPQVST. The signal intensity for these peptides was highly improved after enrichment on iron affinity beads, as shown in Figure 6B.

The finding in Figure 6 suggested that organophosphate labeled albumin peptides could be extracted from human plasma. To test this hypothesis, CPO-treated human plasma (70% labeled albumin) was digested with pepsin and analyzed by MALDI-TOF mass spectrometry. The MS spectrum in Figure 7A for the digest before enrichment shows very low intensities for the masses of interest. In contrast, enrichment on iron affinity beads selectively amplified the signal for the diethoxyphosphate modified peptides VRY₄₁₁TKKVPQVST at 1542 m/z and LVRY₄₁₁TKKVPQVST at 1655 m/z, as well as for the unmodified peptides at 1406 and 1519 m/z (Figure 7B). It was concluded that iron affinity beads could be used to selectively enrich human plasma samples for organophosphate-modified albumin peptides. The special feature of this strategy is that it does not require purification of albumin, but recovers organophosphate-labeled albumin peptides from digests of whole plasma.

PHOS-Select Iron Affinity Gel beads enrich peptides from mouse and guinea pig albumin

Since the mouse and guinea pig are commonly used for studies with organophosphorus toxicants, it was of interest to determine whether albumin from these animals could be monitored for adducts using pepsin digestion and enrichment on iron affinity beads. It was found that peptic peptides of mouse and guinea pig albumin that were equivalent to the tyrosine-411 containing peptide from human albumin could be isolated and enriched with PHOS-Select Iron Affinity beads, using the same protocol that was effective for the peptide from human serum albumin. Please refer to the Supporting Information for details.

Limit of detection—The limit of detection when using 60 μ L beads was 2% of albumin covalently modified on Tyr 411 by CPO. This value is reproducible when measured multiple times for the same sample or when measured from separate preparations. The 2% value was calculated as follows: 2.5 μ L of plasma (40 μ g albumin/ μ L) were diluted to 116 μ L during digestion (0.86 μ g albumin/ μ L); 10 μ L of digest (8.6 μ g albumin) were used for enrichment; the volume of enriched peptides was reduced to 5 μ L (1.7 μ g albumin/ μ L); and 1 μ L (1.7 μ g albumin) was used for MALDI-TOF analysis. Dividing 1.7 μ g albumin by 40 μ g albumin/ μ L in plasma gives 0.043 μ L as the volume of plasma equivalent to the amount of albumin assayed. This calculation assumes no losses during handling. The peptic peptides prepared from this amount of albumin are the maximum that bind to 60 μ L of iron affinity beads. Under these conditions our assay can detect the CPO-modified albumin peptides from a plasma sample in which 2% of the albumin is covalently modified on Tyr 411 (equivalent to 0.034 μ g of albumin).

Prior to enrichment, the labeled peptide is not detectable (Figure 8A) and the unlabeled peptide, at 1518.8 m/z, is barely detectable. Figure 8B shows a MALDI-TOF mass spectrum after enrichment for a sample in which 2% of the albumin is modified by CPO. Peaks for the CPO-peptides at 1541.8 m/z and 1654.8 m/z appear with signal-to-noise ratios of 10-to-1, which was taken as the limit of detection.

The limit of detection when using 500 μ L beads was 0.2% of albumin covalently modified on Tyr 411 by CPO. Figure 8C shows the MALDI-TOF mass spectrum for a sample in which 0.2% of the albumin is modified by CPO; the signal-to-noise ratio for the peak at 1541.8 m/z is 10-to-1.

Why do albumin peptides containing tyrosine 411 selectively bind to iron affinity beads?

Digestion of plasma with pepsin yields dozens of peptides, yet essentially only the tyrosine 411 containing peptides bind to iron affinity beads at pH 11 (see Figure 6). What is special about the tyrosine 411 peptide that allows it to bind to PHOS-Select Iron Affinity Gel? We speculated that free electron pairs from lysine and tyrosine residues, available at pH 11, are employed for binding tyrosine-411 peptides to the Fe^{3+} beads.

To test this hypothesis, we acetylated lysines by treating pure albumin with p-nitrophenyl acetate²⁰. Acetylated residues were identified by mass spectrometry and the effect of acetylation on binding of the tyrosine 411 peptide to Fe^{3+} beads was determined. The results are presented in the Supporting Information. We found that not only the lysines but probably the tyrosine and possibly the peptide N-terminus are involved in chelating the iron. Furthermore, the 3-dimensional structure of the peptide seems to be important for binding.

Discussion

The present report describes a new enrichment strategy for OP-modified albumin peptides. The method was brought to our attention at The 11th International Meeting on Cholinesterases in Kazan, Russia in the year 2012.

Mechanism of albumin peptide binding to Fe³⁺ beads

The Fe³⁺ in PHOS-Select is chelated to the gel by nitrilotriacetic acid that leaves two iron coordination sites available for further interactions. The two available coordination sites provide opportunity for bidentate coordination of the peptide. Based on the “hardness-softness” polarizability concept for interacting ions, Fe³⁺ is considered to be a “hard” Lewis acid i.e. an electron acceptor that associates more strongly with oxygen ligands than with nitrogen or sulfur.²¹ Strong interaction of iron affinity gels with phosphate (phosphopeptides and phospho-proteins) and glutamic acid polymers together with the lack of interaction from monomeric glutamic acid is consistent with the proposal that the Fe³⁺ ion is chelated by two oxygens atoms to effect a strong interaction.²² These oxygen-mediated complexes are formed at relatively low pH (pH 5.5) and are desorbed by raising the pH (to pH 7.0–7.7). The pH dependence of this process is counter to the one required for binding the tyrosine 411 peptides from albumin to Fe³⁺. In the latter case, binding occurs at pH 11 and desorption is accomplished by lowering the pH. It is apparent that a different type of interaction is at play for the tyrosine 411 peptides. We speculate that selective binding of the tyrosine 411 peptides to Fe³⁺ occurs through chelation of lone electron pairs in lysine and tyrosine. Furthermore, not only the primary sequence but also the 3-dimensional structures of the tyrosine 411 albumin peptides seem to be critical for binding to Fe³⁺ beads. See the Supporting Information for details. This is the first example of selective binding of peptides at high pH to Fe³⁺ beads.

Methods to identify OP modified albumin

The first study to report an OP-albumin adduct in humans used pepsin to digest plasma, HPLC to enrich the OP-modified peptide, and MALDI-TOF/TOF mass spectrometry to identify the OP-modified peptide.¹⁴ A more sensitive method for detecting OP adducts on albumin was developed by the Porton Down laboratory.^{9, 10, 23} In that method, plasma or albumin partially purified from plasma is digested with pronase to yield single amino acids. The OP-tyrosine molecule is enriched by solid phase extraction or HPLC, and subjected to liquid chromatography, tandem mass spectrometry where it is detected in a multiple reaction monitoring method. The pronase digestion method has been successfully applied to detection of OP adducts in the blood of guinea pigs, nonhuman primates, mice, and humans.^{9–11, 13, 15} A third method treats OP-modified human plasma with potassium fluoride to release OP from covalent binding to tyrosine and serine and analyzes the diethoxyfluorophosphate product by gas chromatography. This method was used to detect diethoxyfluorophosphate in the plasma of humans poisoned by chlorpyrifos.¹⁵ The present report provides the option of selectively enriching OP-modified albumin peptides along with their unlabeled partners using Fe³⁺ beads. Both the unmodified and OP-modified peptides are enriched, which allows quantitation of the relative amount of OP-labeled albumin.

Why monitor for OP-albumin adducts?

When monitoring human blood for OP exposure, the best protein to study for OP adducts is butyrylcholinesterase. Butyrylcholinesterase (BChE) has broad specificity, reacting with a wide range of OP pesticides and nerve agents. Low OP doses that may not react with other proteins are scavenged by BChE in the skin and bloodstream. Methods to purify BChE from plasma are established, and protocols to prepare samples for mass spectrometry have been developed.^{1, 2, 24} Why assay for OP adducts on albumin?

A major difference between OP adducts on albumin and OP adducts on BChE is that OP adducts on albumin do not age. Soman bound to tyrosine 411 of albumin retains the pinacolyl group. Similarly, the sarin adduct retains the isopropyl group and the cyclosarin adduct retains the cyclohexyl group. By contrast, soman, sarin, and cyclosarin adducts on BChE all age within a few minutes (with soman) to several hours (with sarin and cyclohexyl sarin) to the same methylphosphonate adduct,²⁵ making it impossible to identify the original nerve agent. Thus, OP adducts on albumin are useful for forensics where it is important to know the identity of the nerve agent.

A second difference is the response to treatment with oximes. Poisoned patients are routinely treated with oximes for the purpose of releasing the non-aged OP adduct from acetylcholinesterase. Oximes also release non-aged OP adducts from butyrylcholinesterase. However, oximes do not reverse OP binding to tyrosine. Li et al.¹³ found tyrosine adducts in oxime treated patients who had poisoned themselves with chlorpyrifos.

A third difference is the 20-day half-life of albumin, which compares favorably with the 12-day half-life of BChE in human plasma. OP-adducts on albumin have been detected in blood drawn 49 days after self-poisoning with chlorpyrifos.¹⁵

A fourth difference is the fact that antibodies to OP-adducts on albumin have been successfully made,¹⁸ but attempts to make antibodies to OP-adducts on BChE have failed. The reason for the failure is that the OP-BChE antigen is unstable; OP-modified serine in the BChE peptide undergoes beta-elimination in the circulation of the host animal which eliminates the OP plus a molecule of water to convert OP-serine to dehydroalanine. Methods to avoid beta-elimination have been used to produce antibodies against phosphopeptides, but these methods have not yet been applied to BChE.²⁶ Antibodies to nerve agent-albumin adducts have application in rapid detection devices for exposure to nerve agents.¹⁷

A fifth reason is that OP-adducts on albumin appear to be stable, whereas OP-adducts on BChE of certain animals are unstable. OP-inhibited BChE in the mouse spontaneously recovers activity.²⁷ Moreover, mini pigs have a BChE that self-reactivates quickly after phosphorylation by VX and VR stereoisomers.²⁸ Electric eel acetylcholinesterase self-reactivates after inhibition by methamidophos and phosphoramidates.²⁹

A final reason for analyzing adducts on albumin is that all mammals have albumin in blood, but not all have BChE in blood. For example, cows, goats, and sheep have no plasma BChE, while rats have very low levels of plasma BChE.³⁰

Significance

The method described in the present report has the potential to be useful for monitoring organophosphorus pesticide and nerve agent exposure in animals and humans. Small quantities of blood are needed. The enriched OP-albumin peptide can be analyzed by mass spectrometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations list

CPO	chlorpyrifos oxon
O	<i>O</i> -diethyl <i>O</i> -3, 5, 6-trichloro-2-pyridyl phosphate
CHCA	α -cyano-4-hydroxycinnamic acid
OP	organophosphorus toxicant
MALDI-TOF/TOF	matrix assisted laser desorption ionization tandem time-of-flight mass spectrometry
CID	collision induced dissociation
DE	delayed extraction
m/z	mass divided by charge
BChE	butyrylcholinesterase

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Figure 1.

MALDI-TOF mass spectra of pepsin digested pure human albumin before and after enrichment on iron affinity gel beads. A) Pepsin digest of human albumin before enrichment. B) The same digest after enrichment on iron affinity gel beads. The peaks at 1405.9 and 1519.0 m/z are for peptides VRY₄₁₁TKKVPQVST and LVRY₄₁₁TKKVPQVST (accession # gi: 122920512 for human albumin).

Figure 2.

A) CPO reacts with albumin by donating its diethoxyphosphate group to tyrosine 411, resulting in an added mass of 136 Da. B) Soman reacts with albumin to yield pinacolyl methyl phosphonate-tyrosine 411 with an added mass of 162 Da.

Figure 3. MALDI-TOF mass spectra of CPO-treated pure human albumin digested with pepsin before and after enrichment on iron affinity gel. A) Pepsin digest of CPO-human albumin before enrichment. B) The same digest after enrichment on iron affinity gel beads.

Figure 4. MALDI-TOF mass spectra of soman-treated pure human albumin digested with pepsin, before and after enrichment on iron affinity gel. A) Pepsin digest of soman-human albumin before enrichment. B) The same digest after enrichment on iron affinity gel beads.

Figure 5. Tyrosine 411 in human albumin is modified by CPO or soman. A) MALDI MS/MS spectrum of parent ion 1405.9 m/z from untreated albumin identifies the peptide as VRYTKKVPQVST. B) MS/MS spectrum of parent ion 1541 m/z from CPO-treated albumin identifies tyrosine 411 as the modified residue. C) MS/MS spectrum of parent ion 1567.0 m/z from soman-treated albumin identifies tyrosine 411 as the modified residue. Peaks labeled -84 (minus 84) have lost the pinacolyl group.

Figure 6. MALDI-TOF mass spectra of pepsin digested human plasma (diluted 40-fold) before and after enrichment on iron affinity gel beads. A) Pepsin digest of human plasma before enrichment. B) The same digest after enrichment on iron affinity gel beads selectively increases the signal intensity of peptides VRY₄₁₁TKKVPQVST and LVRY₄₁₁TKKVPQVST.

Figure 7. MALDI-TOF mass spectra of human plasma treated with CPO and digested with pepsin, before and after enrichment on iron affinity gel beads. A) Pepsin digest of CPO-human plasma before enrichment. B) The same digest after enrichment on iron affinity gel beads. The asterisk indicates that tyrosine 411 of human albumin is the residue modified by diethoxyphosphate.

Figure 8.

Limit of detection. MALDI-TOF mass spectra of a pepsin digest of human plasma covalently modified by CPO. A) Pepsin digest before enrichment on PHOS-select beads. B) The same digest after enrichment on 60 μ l beads, where the limit of detection is 2% CPO-albumin in 0.043 μ l plasma. C) After enrichment on 500 μ l beads, the limit of detection is 0.2% CPO-albumin in 0.43 μ l plasma. The asterisk indicates that tyrosine 411 of human albumin is the residue modified by diethoxyphosphate.