HHS Public Access

Author manuscript

Chemosphere. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Chemosphere. 2018 May ; 199: 154–159. doi:10.1016/j.chemosphere.2018.02.003.

Covalent Binding of the Organophosphate Insecticide Profenofos to Tyrosine on α- and β-Tubulin Proteins

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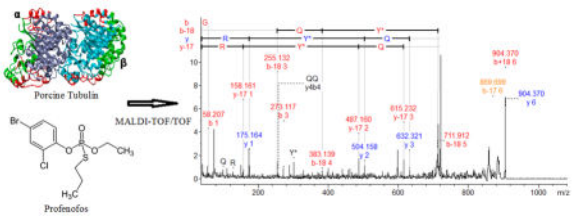
Abstract

Organophosphorus (OP) compounds can bind covalently to many types of proteins and form protein adducts. These protein adducts can indicate the exposure to and neurotoxicity of OPs. In the present work, we studied adduction of tubulin with the OP insecticide profenofos *in vitro* and optimized the method for detection of adducted peptides. Porcine tubulin was incubated with profenofos and was then digested with trypsin, followed by mass spectrometry identification of the profenofos-modified tubulin and binding sites. With solvent-assisted digestion (80% acetonitrile in digestion solution), the protein was digested for peptide identifications, especially for some peptides with low mass. The MALDI-TOF and LC-ESI-TOF analysis results showed that profenofos bound covalently to Tyr83 in porcine α-tubulin (TGTY*$_{83}$R) and to Tyr281 in porcine β-tubulin (GSQQY*$_{281}$R) with a mass increase of 166.02 Da from the original peptide fragments of porcine tubulin proteins. Tyrosine adduct sites were also confirmed by MALDI-TOF/TOF analysis. This result may partially explain the neurotoxicity of profenofos at a low dose and a prolonged period of exposure.

Graphical abstract



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Conflict of Interest

The authors declare that there is no conflict of interests.

Keywords

Adduct; Exposure; LC-Q-TOF-MS; MALDI-TOF/TOF; Organophosphate; Profenofos; Tubulin

1. Introduction

Organophosphates (OPs) are commonly used as pesticides and flame retardants (Kazemi and Tahmasbi, 2012; Campos et al., 2016; He et al., 2016; Schreder et al., 2016; Voorhees et al., 2017). Exposure to OP insecticides can lead to acute or chronic poisoning. The acute toxicity of OP insecticides is mainly due to inhibition of acetylcholinesterase (AChE) (Colovic et al., 2013; Kushwaha et al., 2016). However, AChE inhibition alone cannot explain a wide range of OP neurotoxicities, which includes deficits in cognition, learning and memory, depression, psychotic symptoms and other neurotoxicity when victims were exposed to OP insecticides at low doses and over a prolonged period (Casida and Quistad, 2004; Beseler and Stallones, 2008; Briceno et al., 2016; Campos et al., 2016; Voorhees et al., 2017). One hypothesis to explain this long-lasting neurotoxicity from low doses of OP insecticides is that the OPs covalently modify specific amino acid residues of some protein surface regions in the axonal transport system and, therefore, result in protein adduction. When adduct formation impairs important protein structures, the protein functions such as catalytic activity and ion translocation will be affected and toxicity occurs (Marsillach et al., 2013; Yang and Bartlett, 2016; Mangas et al., 2017).

While acute toxicity of the OPs has been widely studied and long been known, studies on the mechanism of long-lasting OP neurotoxicity are relatively rare. The determination of protein adducts is a relatively new research area (Tornqvist et al., 2002; Yang and Bartlett, 2016; Sabbioni and Turesky, 2017; Chu et al., 2017). OP adducts with serum albumin and hemoglobin were commonly studied as biomarkers of effect and exposure (Casida and Quistad., 2004; Peeples et al., 2005; John et al., 2010; Crow et al., 2014, Yang and Bartlett, 2016; Sabbioni and Turesky, 2017). Cytoskeletal proteins including tubulin and transferrin have been implicated in the *in vivo* or *in vitro* neurotoxicity of OPs (Abou-Donia, 2003; Grigoryan et al., 2008; Jiang et al., 2010; Marsillach et al., 2013; Sun et al., 2016).

Tubulins are present in all eukaryotic cells as the constituents of microtubules that are major components of the cytoskeleton network participating in many cellular functions, such as cell division and intracellular trafficking (de Forges et al. 2012; Breuss and Keays 2014; Lancaster and Baum, 2014). Microtubules are assembled from heterodimers of α- and β-tubulin, each with an approximate molecular weight of 50k Da and with very similar amino acid compositions (Krauhs et al., 1981; Nogales et al., 1998). Microtubules are integral components of transport systems as well as important mechanical elements that contribute to cell shape and stiffness. Kinesin motor proteins in the cell bind to a variety of cargo, including vesicles, and utilize the chemical energy from ATP hydrolysis to transport these cargos along microtubules of the cytoskeletal highway (Ross et al., 2008; Malcos and Hancock, 2011). These microtubules have dynamic instability; there is coexistence of assembly and disassembly at their ends with addition and loss of tubulin subunits (Cassimeris et al., 1987; Malcos and Hancock., 2011). Since microtubules are required for

transport of nutrients from the nerve cell body to the nerve synapse, disruption of microtubule function could explain the neurotoxicity associated with OP exposure (Prendergast et al., 2007; Jiang et al., 2010; Briceno et al., 2016).

The goal of the present work was to obtain information about whether the OP profenofos can covalently modify specific amino acid residues of tubulin and where specifically the adduct sites are in the protein surface regions. Profenofos is an OP insecticide widely used on a variety of crops including cotton and vegetables, such as maize, potato, soybean, and sugar beet. In the United States, it is used exclusively on cotton and is primarily used against lepidopteran insects (US EPA, 2000; Kushwaha et al., 2016). Tubulin was selected for this study because covalent binding of OPs to tubulin is a potential mechanism of neurotoxicity (Jiang et al., 2010; Kushwaha et al., 2016). To render it possible to identify tubulin as a target protein of profenofos binding, an *in vitro* method was optimized to identify tubulin adducts with profenofos by matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF) and liquid chromatograph-quadrupole time-of-flight mass spectrometry (LC-Q/TOF).

2. Material and methods

2.1. Chemicals

Tubulin protein (> 99 % pure) from porcine brain and guanosine 5-triphosphate sodium salt (GTP) were purchased from Cytoskeleton, Inc. (Denver, CO, USA). This tubulin preparation contains both α- and β-tubulin. Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). A stock solution of porcine brain tubulin (0.6 mg/mL) in 15 mM ammonium bicarbonate (NH_4HCO_3) was prepared, and then was snap frozen and stored at −80°C until use.

Profenofos was purchased from Sigma-Aldrich (St. Louis, MO, USA). α-Cyano-4-hydroxycinnamic acid (HCCA) used as MALDI matrix and peptide calibration standard II were from Bruker (Billerica, MA, USA). DL-dithiothreitol (DTT) was from Acros Organics (New Jersey, USA). Vivaspin™ 500 centrifugal concentrators VS0102 (cut-off 10 kDa) were obtained from Sartorius Stedim North America, Inc. (New York, USA). HPLC grade acetonitrile (ACN), dimethyl sulfoxide (DMSO), ammonium hydroxide (NH_4OH), ammonium bicarbonate (NH_4HCO_3) and formic acid were from Fisher Scientific (Waltham, MA, USA). Water was purified on a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Sample preparation and protein enzymatic cleavage

A 50 μL aliquot of porcine brain tubulin solution (0.6 mg/mL in 15 mM NH_4HCO_3) was spiked with 0.5 μL GTP (100 mM). A 2.5 μL aliquot of profenofos solution (9.6 mM in DMSO) was added into the tubulin solution, followed by incubation at 37 °C for 24 h under gentle shaking. The mixture was then subjected to ultrafiltration using a Vivaspin 500 centrifugal concentrator, which was pre-washed three times with 400 μL of water, to concentrate the protein and separate unreacted profenofos from the mixture solution at 14000*g*. After the filtrate was discarded, the residue was washed seven times with 400μL of 25 mM (NH_4HCO_3). The concentrated tubulin protein solution (aboutμL) 25 was transferred

into a vial. The concentrator was rinsed with 25 μL of NH_4HCO_3 buffer and the rinsates were combined with the tubulin solution fractions. An aliquot of 200 μL ACN was added to the solution and then 6 μL of trypsin (0.1 μg/μL) was added. The trypsin digestion was performed at 37 °C for one hour. AμL10-aliquot of 1 % formic acid was added into the solution to inhibit further trypsin activity.

A blank control sample with no profenofos fortified was prepared in the same manner as described above. In this study all assays were performed in triplicate. All samples were stored at −80 °C until analysis.

2.3. MALDI-TOF/TOF analysis

The tryptic peptide samples were analyzed an Ultraflex by^{III} MALDI-TOF/TOF mass spectrometer (Bruker, Billerica, MA, USA) equipped with a Smartbeam laser system. Mass spectra of the peptides were acquired in a reflector positive mode. Samples were spotted using the overlay method as follows. A 1-μL aliquot of digest sample was applied onto a stainless-steel target plate, air-dried, and overlaid with 1 μL HCCA (10 mg/mL in a solvent mixture of acetonitrile/water/TFA, 50v/47.5v/2.5v) solution and allowed to air-dry at room temperature. The following parameters were set for peptide detection: ion source 1, 25.00 kV; ion source 2, 21.65 kV; lens, 9.25 kV; reflector, 26.30 kV; reflector 2, 13.85 kV. For MS/MS experiments, LIFT mode was used under the following parameters: ion source 1, 8.0 kV; ion source 2, 7.2 kV; lens, 3.6 kV; reflector, 29.63 kV; reflector 2, 13.91 kV; LIFT 1, 19.08 kV, LIFT 2, 3.71 kV. The measurements were performed in a mass range from *m/z* 300–4000 Da with a maximum mass deviation of 0.3 Da. Three thousand laser shots were accumulated in 200 shot increments and resulted in the final mass spectrum. External mass calibration was carried out with the peptide calibration standard II from Bruker. FlexControl software (v.3.4) was used for controlling the system and mass searching was performed using FlexAnalysis (v.3.4) and BioTools (v.3.2).

2.4. LC-ESI-Q-TOF analysis

To confirm the results from the MALDI-TOF-MS analysis, he digested tubulin peptide samples were analyzed with an Agilent 1200 LC system coupled to an Agilent 6520A quadrupole time-of-flight mass spectrometer (LC-Q-TOF-MS) system (Agilent Technologies, Mississauga, ON, Canada). The remaining sample solution after MALDI-TOF/TOF analysis was transferred into a sample vial with 200 μL of 50% ACN aqueous solution. The tryptic peptides were separated on a Kinetex XB-C18 column (100 mm × 2.0 mm, 1.7 μm particle size) (Phenomenex Co., CA, USA). A 10-μL aliquot of sample was injected. The LC mobile phase consisted of A: 0.1 % formic acid in water and B: 0.1% formic acid in ACN. The flow rate was set to 0.3 mL/min and the temperature of the column compartment was at 40 °C. The LC gradient started at 5% B, increasing to 40% B in 40 min, increasing to 95% in 42 min and was held for 18 min. Thereafter, the mobile phase composition was returned to initial conditions and the column was allowed to equilibrate for 15 min prior to the next injection. The electrospray ionization source was operated in positive mode (ESI+). The capillary voltage was set to 5500 V, the fragmentor and skimmer voltages were 170 V and 70 V, respectively. Nitrogen gas was used as drying and nebulizing gases. The gas temperature was 350 °C. Dry gas flow rate was 5 L/min. Nebulizer pressure

was 20 psi. Full-scan data acquisition was performed by scanning from *m/z* 70 to *m/z* 3200. The TOF-MS was tuned and calibrated with mixture calibration solution from Agilent Technologies. Internal mass calibration was carried out with reference masses of *m/z* 121.050873 and 922.009798. Data were acquired with Mass Hunter 6.0.

3. Results and Discussion

3.1. Adduct formation and sample preparation

A common scenario for a protein adduct assay and subsequent analysis by MS includes (1) formation of adducts between protein and a xenobiotic at key amino acids, (2) removal of excess xenobiotics, and (3) identification and characterization of the adducted protein by bottom-up or top-down mass spectrometric approaches (Yang and Bartlett, 2016; Sabbioni and Turesky, 2017). The most common method is still bottom-up proteomics, because it is more compatible with generally available MALDI-TOF-MS and LC-Q-TOF-MS in comparison with top-down approach (Yang and Bartlett, 2016; Sabbioni and Turesky, 2017; Chu et al., 2017). In bottom-up methods, the xenobiotic treated protein is digested into peptides that can be efficiently analyzed with a wide range of LC–MS or MALDI-TOF MS instruments. However, it is noteworthy that bottom-up mass spectrometric approaches require a number of pretreatment steps in order to get from the protein to the peptide level. The results from a bottom-up approach are thus affected by sample preparation and treatment which includes denaturation and disulfide bond reduction and alkylation, to name a few. Grigoryan et al. (2009a–c) published several research papers about mass spectral identification of covalent binding of tubulin to chlorpyrifos oxon (CPO) as well as for some other OPs. The proteins were first denatured in boiling water, followed by removal of excess OPs via dialysis. This sample preparation was also adopted in other studies (Sun et al., 2016). However, the protein in the denatured state is very different from its native state (folded state). The active sites in a denatured protein differ from those in a native protein and they might react with the test chemical at high temperature during denaturation process (100 °C). This may explain the reason why many more adduct sites were observed in their experiments than those observed by other researchers in *in vitro* or *in vivo* experiments (Jiang et al., 2010). It was reported that dialysis would result in loss of up to 95 % of the tubulin protein due to sticking to the dialysis membrane (Jiang et al., 2010). Therefore, in the present study, ultrafiltration was used to separate excess profenofos and concentrate the sample.

3.2 Enzymatic cleavage

Prior to MS analysis, efficient digestion is crucial for successful identification of the protein sites where xenobiotics covalently bind. In the present method, trypsin was used for proteolytic cleavage of tubulin because trypsin is the most commonly used protease for generation of MS amenable peptides. Trypsin has a defined specificity; it cleaves the peptide bonds that are C-terminal to the basic residues Lys and Arg, except when followed by Pro, which is very suitable for tubulin digestion followed by MS analysis (Grigoryn et al., 2008, 2009a, Jaing et al., 2010; Albert-Baskar et al., 2015; Yang and Bartlett, 2016). Although trypsin digestion has been well described in the literature, given the wide range of available reagents, the detailed methods of digestion vary greatly among laboratories. Conventional

trypsin in-solution digestion was initially tried in our preliminary experiments. In this process, tubulin (0.6 mg/mL in 15 mM NH_4HCO_3) was denatured in boiling water for 10 min, reduced by DTT and alkylated by IAA, and the remaining IAA was then removed by addition of DTT. After the tubulin was digested with 0.6 μg trypsin at 37 °C overnight, the digested sample was concentrated to about 20 μL and then desalted with a ZipTip-C18 pipette tip. The MALDI-TOF results showed that although the sequence coverage could reach to 57% and 76% for α-tubulin and β-tubulin, respectively, some of important low mass peptide peaks, such as $TGTY_{83}P$ and $GSQQY_{281}R$, were missing in the mass spectrum. Correspondingly, no protein adduct could be detected in the samples.

Some accelerating strategies for protein digestion, such as using solvent effects and ultrasonic energy to digestion, have been recently reported (Hustoft et al., 2011). It was reported that solvent-assisted digestion could reduce the digestion time and risk of sample loss (Russell et al., 2001; Strader et al., 2006). Because the protein can be denatured by organic solvents in solvent-assisted digestion, thermal denaturation, which often results in sample loss due to precipitation, is unnecessary. In our experiment, aqueous ACN solution was used in solvent-assisted digestion and varying percentages of ACN in the digestion solution were tested to optimize trypsin digestions. The results showed that as high as 80% of ACN in the solution could be used and sequence coverage could still reach to 46% and 66% for α-tubulin and β-tubulin, respectively. This result is in accordance with that reported elsewhere (Russell et al., 2001; Strader et al., 2006). Solvent-assisted digestion with ACN provided more low mass peptides. In our experiments, the important low mass peptide peaks, such as $TGTY_{83}P$ and $GSQQY_{281}R$ could be detected in trypsin solvent-assisted digested tubulin solution. Solvent-assisted digestion yielded much higher ion signals in comparison to digestion in aqueous solution. The digestion solution can be directly analyzed by LC–MS or MALDI-TOF, and concentration and desalting processes are unnecessary.

3.3. Determination of tubulin phosphorylation by profenofos using MALDI-TOF/TOF

The peptides from tubulin digested by trypsin were subsequently analyzed with MALDI-TOF MS. Figure 1 (a) and (b) show the MALDI-TOF mass spectra of trypsin-digested porcine tubulin that was incubated without (blank control sample) and with profenofos, respectively. In comparison with the blank control sample, the peak at *m/z* 904.388 (theoretical *m/z* 904.3752) in profenofos-fortified samples corresponded to phosphorylated adduct peptide of $GSQQY_{281}R$ from β-tubulin with a mass increase of 166.02 Da. By MALDI-TOF/TOF analysis (in the LIFT experiment), the relevant fragments of its precursor ions of *m/z* 904.388 clearly showed that profenofos covalently reacted with the Tyr_{281} residue (Figure 2), likely via the reaction shown in Figure 3. This result was consistent with earlier studies for other OPs (Grigoryan et al., 2008; Jiang et al., 2010).

3.4. Determination of tubulin phosphorylation by profenofos using LC-ESI-Q-TOF

Although MALDI-TOF-MS is a quick and reliable method for the identification of peptides, and it can give a cleaner ion profile of peptide mixtures directly even without pre-separation (Kafka et al., 2011; Nomura, 2015), the interference between different peptides in the complex mass spectrum may result in false positive results. Considering the relatively low percentage of covalently modified amino acid residues in the target protein, this interference

problem may be even more serious. LC-ESI-Q-TOF-MS provides a versatile method for the detection of protein adducts because LC can separate peptides and TOF-MS can detect multiply charged peptide ions with high mass resolution, high accuracy and relatively high sensitivity (Kollipara et al., 2011; Yang and Bartlett, 2016). Therefore, LC-ESI-Q-TOF was used in our experiment to confirm the results from MALDI-TOF-MS. Figure 4 shows a mass spectrum of a chromatographic peak at retention time of 14.16 min. In the mass spectrum, *m/z* 904.3717 9 ($[M+H]^+$) and *m/z* 452.6893 ($[M+2H]^{2+}$) corresponded to the phosphorylated adduct peptide of $GSQQY_{281}R$ from β-tubulin, which confirms the result from MALDI-TOF-MS analysis.

There was another phosphorylated adduct peptide detected by the LC-ESI-Q-TOF method. Figure 5 shows the mass spectrum of the chromatographic peak at retention time of 15.11 min, and *m/z* 763.3194 ($[M+H]^+$) and *m/z* 382.1641 ($[M+2H]^{2+}$) corresponded to the profenofos adducted peptide of $TGTY_{83}R$ (theoretical *m/z* 763.3213 and *m/z* 382.1643). This adduct peptide could not be detected by MALDI-TOF-MS, but it was detectable with LC-ESI-Q-TOF. This might be resulted from high ionization efficiency of this peptide by ESI and chromatographic separation which reduced interferences.

The MALDI-TOF and LC-ESI-Q-TOF analysis results showed that profenofos binds covalently to Tyr83 in porcine α-tubulin and to Tyr281 in porcine β-tubulin. These tyrosine adduct sites were also the most reactive sites in bovine and mouse tubulin (Grigoryn et al., 2009c, Jiang et al., 2010). Crystal structure of tubulin heterodimers showed that Tyr83 and Tyr281 are well exposed to solvent and this might partly explain their high reactivity with OPs (Nogales et al., 1998).

We also tested if the results were affected by reduction and alkylation prior to trypsin digestion. The tubulin sample treated with profenofos (after ultrafiltration) was denatured, reduced DTT and alkylated by IAA, and then solvent-assisted digested by trypsin. The MS by analysis results were the same as that described previously.

In conclusion, our results showed that profenofos can covalently react with tubulin. Tubulins are enriched in neurons and modification of these cytoskeletal proteins can affect their interaction, polymerization, and stabilization. Ultimately, this could affect microtubule stability and could disrupt nutrient transportation (Abou-Donia, 2003; Gearhart et al., 2007; Prendergast et al., 2007). Although the toxic and adverse behavioral effects of exposure to profenofos likely involves a broad array of effects on cytoskeletal components, its adduction with tubulin may give some clues to explain the neurotoxicity of profenofos and other OPs at low doses for a prolonged period of exposure.

Acknowledgments

This work was supported in part by grant G12 MD007601 from the National Institutes of Health Research Centers in Minority Institutions Program, a subcontract with the Western Center for Agricultural Health and Safety (NIOSH grant 2U54OH007550), and by the USDA National Institute of Food and Agriculture, Hatch project HAW5032-R, managed by the College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa.

Abbreviations

OPs	Organophosphorus compounds
HCCA	α-cyano-4-hydroxycinnamic acid
ACN	acetonitrile
DMSO	dimethyl sulfoxide
AChE	acetylcholinesterase
MALDI-TOF/TOF	matrix-assisted laser desorption/ionization time-of-flight/ time-of-flight mass spectrometry
LC-Q-TOF-MS	liquid chromatography quadrupole time-of-flight mass spectrometry
DTT	DL-dithiothreitol
LC-ESI-TOF	liquid chromatography electrospray ionization time-of-flight mass mass spectrometry
CPO	chlorpyrifos oxon
GTP	guanosine 5-triphosphate

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Highlights

- Profenofos can form adducts with porcine tubulin.
- Profenofos binds covalently to Tyr83 of α-tubulin and to Tyr281 of β-tubulin.
- An *in vitro* method can detect tubulin-profenofos adducts and their adduct sites.
- Solvent-assisted trypsin digestion is simple and effective for adducted peptide preparation.

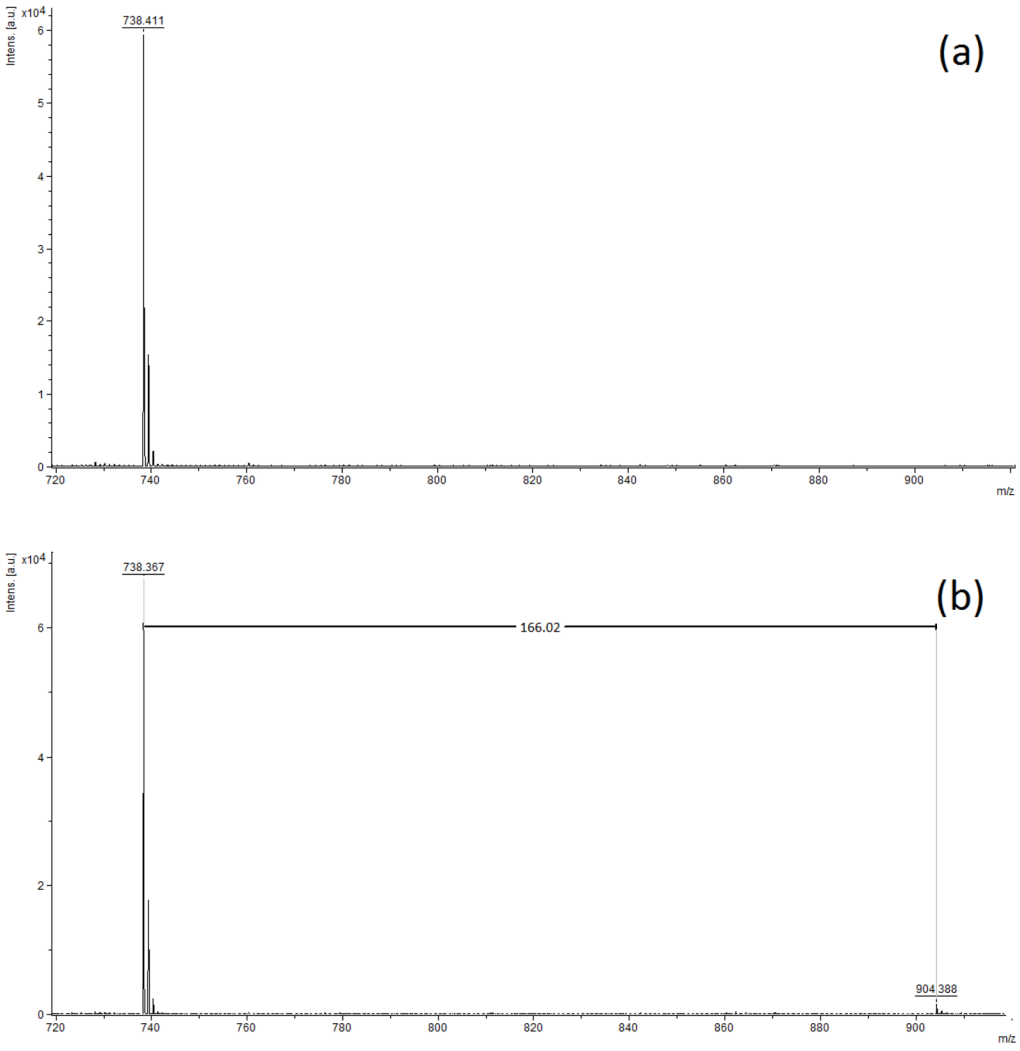

Fig. 1.

MALDI-TOF mass spectra of trypsin-digested porcine tubulin incubated without profenofos (a) and with profenofos (b). The resulting adduct peptide ion at *m/z* 904.388 originated from *m/z* 738.367 ($GSQQYR_{282}$) with mass increase of 166.02 Da.

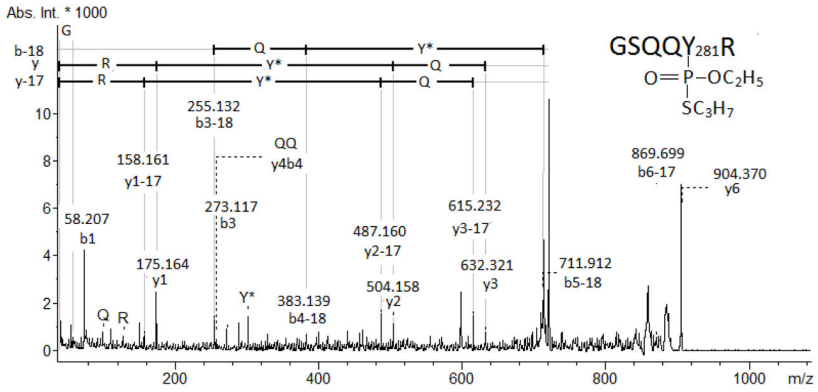

Fig. 2.

MALDI-TOF/TOF MS/MS spectrum of the precursor adduct peptide ion at *m/z* 904.388. Fragmentation shows that profenofos covalently reacted with the Tyr281 residue in the peptide $GSQQY_{281}R$.

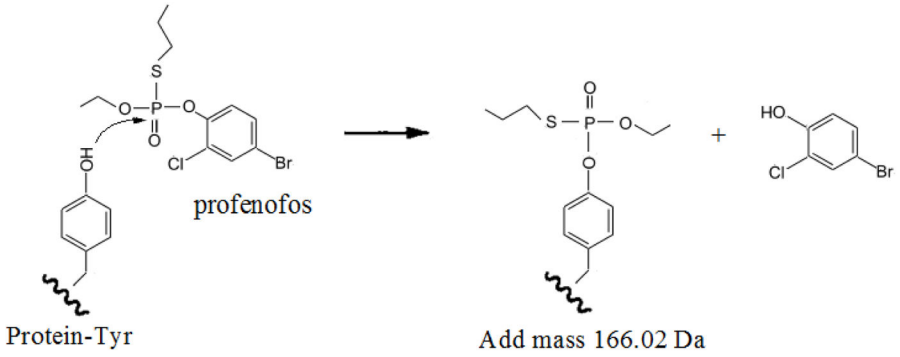

Fig. 3.
A schematic diagram of reaction of profenofos with a tyrosine residue.

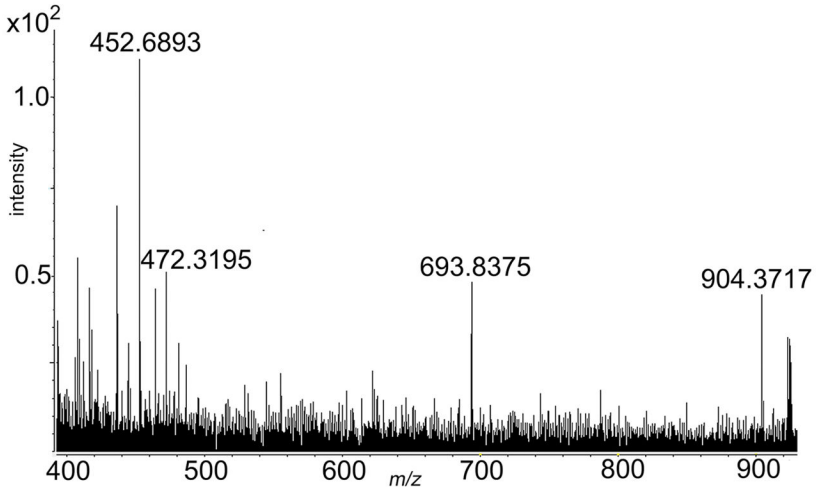

Fig. 4.

LC-ESI-TOF mass spectrum of the chromatogram peak with a retention time of 14.16 min. Ions of *m/z* 904.3717 ($[M+H]^+$) and *m/z* 452.6893 ($[M+2H]^{2+}$) correspond to the phosphorylated adduct of the peptide $GSQQY_{281}R$ from β-tubulin, and confirm the result from MALDI-TOF-MS analysis (see Figure 2).

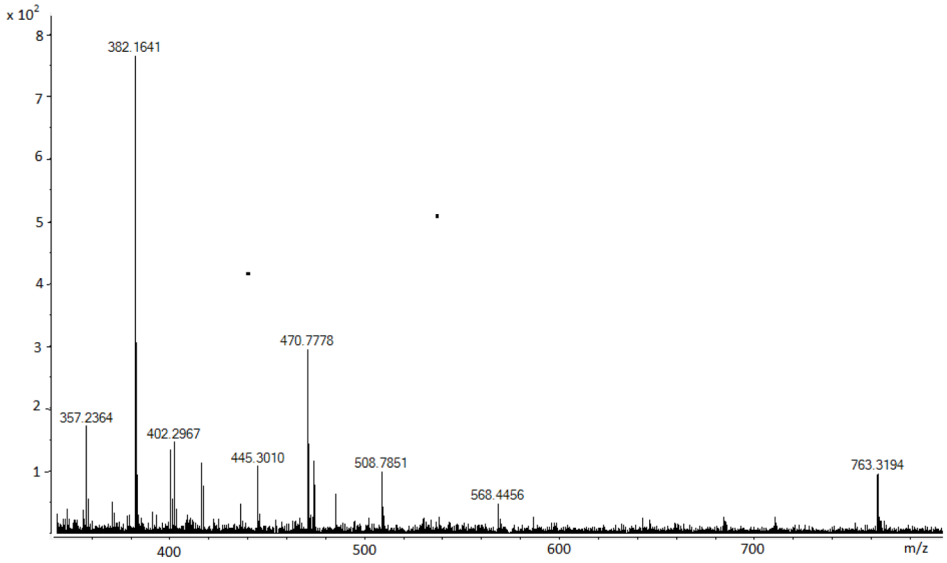

Fig. 5.

LC-ESI-TOF mass spectrum of the chromatogram peak with a retention time of 15.11 min. Ions of *m/z* 763.3194 ($[M+H]^+$) and *m/z* 382.1641 ($[M+2H]^{2+}$) corresponded to the profenofos adducted peptide $TGTY_{83}R$ from α-tubulin.