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# Analysis of organophosphorus compound adducts of serine proteases by liquid chromatography–tandem mass spectrometry

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**Abstract**

In order to confirm that diisopropylfluorophosphate (DFP) phosphorylates the active site serine residue in α-chymotrypsin, a peptide containing the phosphorylated active site was analyzed by liquid chromatography (LC)–electrospray mass spectrometry (ESI-MS). After reduction with dithiothreitol and subsequent alkylation with acrylamide, α-chymotrypsin was digested by treatment with trypsin. Tryptic digest was subjected to LC–ESI-MS. Nearly all the peptide fragments were identified by comparison with fragments predicted from as tryptic digest of α-chymotrypsin. From the tryptic digest of native α-chymotrypsin, a doubly protonated peptide peak which corresponded to the peptide fragment containing the active site serine residue was detected on a selected ion chromatogram at $m/z$ 1265.0, and the sequence was determined to be ''DAMICAGASGVSSCMGDSGGPLVCK''. From the tryptic digest of DFP-inhibited α-chymotrypsin, the doubly protonated peptide peak was detected on a selected ion chromatogram at $m/z$ 1347.0. The difference in mass number (82 in a doubly charged ion) of active site peptide fragments between the native and DFP inhibited α-chymotrypsins was assumed to be the result of phosphorylation of the serine residue with a diisopropylphosphoryl moiety. A total of +164 Da mass shifts of y-series fragment ions from the $y_8$ to $y_{21}$ positions in the active site peptide of the DFP inhibited α-chymotrypsin was observed, in comparison with the native α-chymotrypsin. Thus, the phosphorylation site in α-chymotrypsin could be unequivocally identified to be at the serine residue which is located at position 47, from the N-terminus of the α-chymotrypsin C-chain.


*Keywords:* Serine protease; Organophosphorus

## 1. Introduction

Serine hydrolases, such as chymotrypsin (EC 3.4.21.4) and acetylcholinesterase (EC 3.1.1.7), are proteolytic and esteratic enzymes the active sites of which contains a serine residue and related charge relay systems [1]. Organophosphorus compounds irreversibly inhibit serine hydrolase activity, via the phosphorylation of the active site serine residue [2]. A conventional biochemical approach determined the active site serine residue using [$^{32}$P] labeled organophosphorus compounds. Enzymes were phosphorylated with radio-labeled inhibitors, and enzymatically digested to a series of peptides, and the subsequent fragments were separated by liquid chromatography, subjected to Edman sequencing to identify the phosphorylation site by monitoring the release of radioactivity during the Edman cycles [3,4].

Recently, sophisticated mass spectrometric techniques have been developed for the determination of

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the primary structure of proteins, ever though they are modified post-translationally, such as by phosphorylation [5] and glycosylation [6,7]. In particular, using a new type of hybrid mass spectrometer which combines a quadrupole analyzer and a time-of-flight (TOF) mass analyzer, biopolymers have been structurally characterized after proteolytic digestion [8,9].

In this paper, using α-chymotrypsin (EC. 3.4.21.4) as an example of a serine hydrolase and diisopropylfluorophosphate (DFP) as an example of organophosphorus inhibitors, liquid chromatography–mass spectrometry (LC–MS) was applied to the elucidation of the active site phosphorylation site.

## 2. Experimental

### 2.1. Reagents

α-Chymotrypsin from bovine pancreas was purchased from Merck (Darmstadt, Germany). DFP was obtained from Sigma (St. Louis, MO). Other chemicals were from Wako Pure Chemical (Osaka, Japan). All buffers were prepared with distilled water. DFP is a highly toxic chemical and necessary safety precautions must be taken while handling this reagent.

### 2.2. Formation of DFP adduct of α-chymotrypsin

A 2 μl volume of 40 m*M* DFP in acetonitrile was added to 100 μl of an α-chymotrypsin solution (10 μg/μl in 20 m*M* sodium phosphate (pH 7.2)), and the mixture was incubated at 37 °C for 1 h in a water bath. For a control experiment, 2 μl of acetonitrile was added in place of the DFP solution.

Protease activity of the DFP inhibited enzyme was assayed by measurement of the hydrolysis of *N*-α-benzoyl-L-tyrosine ethyl ester (BTEE) as an increase in absorbance at 254 nm [10].

### 2.3. Tryptic digestion

A 100 μl volume of 1 *M* Tris–HCl/8 *M* urea/80 m*M* dithiothreitol (DTT) solution (pH 8.5) was added to the treated α-chymotrypsin solution as described in Section 2.2, and the mixture incubated at 50 °C in the dark for 1 h. After this procedure, a 100 μl volume of 7 *M* acrylamide in water was added, and the mixture incubated at 25 °C in the dark for 1 h. The samples were desalted and concentrated to 50 μl using a Centricon 30 (Millipore, Bedford, USA).

The reduced, alkylated α-chymotrypsin solution was mixed with a 20 μl volume of 500 m*M* ammonium bicarbonate in water (pH 8.0), and adjusted to 200 μl with distilled water. A 2 μl volume of trypsin solution (10 μg/μl) was added to the above solution, and the mixture was incubated at 37 °C for 10 h. The digestion was terminated by the addition of 2 μl of formic acid.

### 2.4. LC–MS analysis

A Micromass Q-TOF2 mass spectrometer (Micromass, Altrincham, UK), equipped with a nebulized electrospray z-spray source was use for the LC–MS analysis. The mass spectrometer was coupled with an Agilent 1150 HPLC system (Agilent, CA, USA).

A μs-CrestPak $C_{18}$S column (150 mm length, 1.5 mm diameter, JASCO, Tokyo, Japan) was use as separation column. The stationary phase was equilibrated with 95% A/5% B solvent (solvent A, 0.1% formic acid in water; solvent B, 0.086% formic acid/80% acetonitrile in water). Elution was performed at a flow-rate of 0.2 ml/min with a linear gradient from 95% A/5% B to 100% B over 60 min with an initial 5 min hold. The absorbance of the column eluate was monitored at 215 nm. A 20 μl volume of sample was injected to the LC in each analysis. The entire was introduced into the electrospray interface.

The capillary voltage, cone voltage and microchannel plate (MCP) detector voltage were set to 3000, 35 and 2200 V, respectively. The collision energy was set at 10 V. Desolvation was carried out by a stream of nitrogen at 250 °C. The instrument operation, data acquisition and data analysis were performed using MassLynx 3.2 software (Micromass), on a Windows NT workstation.

In the MS/MS measurement, mass spectrometric parameters were adjusted as follows: the MCP voltage was set to 2500 V, and appature 2 was 18 V.

The collision energy was optimized from 20 to 45 V for each peptide fragment.

## 3. Results

### 3.1. Native α-chymotrypsin

α-Chymotrypsinogen is a polypeptide with a molecular mass of approximately 25 000, and contains five intermolecular disulfide bridges [11]. α-Chymotrypsinogen is converted to α-chymotrypsin by the proteolytic action of trypsin, which releases two dipeptides ($Leu^{13}$-$Ser^{14}$, $Thr^{147}$-$Asn^{148}$) [12,13]. Table 1 shows the primary structure of the three peptide chains (A, B and C chain) of α-chymotrypsin. Table 2 shows the deduced peptide fragments of a tryptic digest of reduced/acrylamide-modified α-chymotrypsin. The fragmentation information was obtained from the primary structure in the literature [11] and confirmed by ''MS Digest'' software (UCSF Mass Spectrometry Facility, USA) [14]. In this table, the peptide fragments which are smaller than a tetra-peptide are not shown.

Fig. 1 shows the ultraviolet (UV) absorbance, total ion and selected ion chromatograms of a peptide digest of native α-chymotrypsin. On the extracted ion chromatogram of $m/z$ 1265.0 which corresponds to the doubly protonated peptide fragment containing the active site serine residue, one major peak was obtained having a retention time of 26.9 min. In the mass spectrum of this peak (Fig. 2), doubly protonated ($m/z$ 1265.0) and triply protonated ($m/z$ 843.0) ions were obtained, although ions at $m/z$ 850.5 and at $m/z$ 1700.0 were observed as major peaks, and are assumed to be derived from unseparated overlapping peptides. The sequence of this peak was estimated to be ''LQQASLPLLSNTNCK'' (Table 2).

All the other peptide fragments of native α-chymotrypsin listed in the Table 1 were also iden-

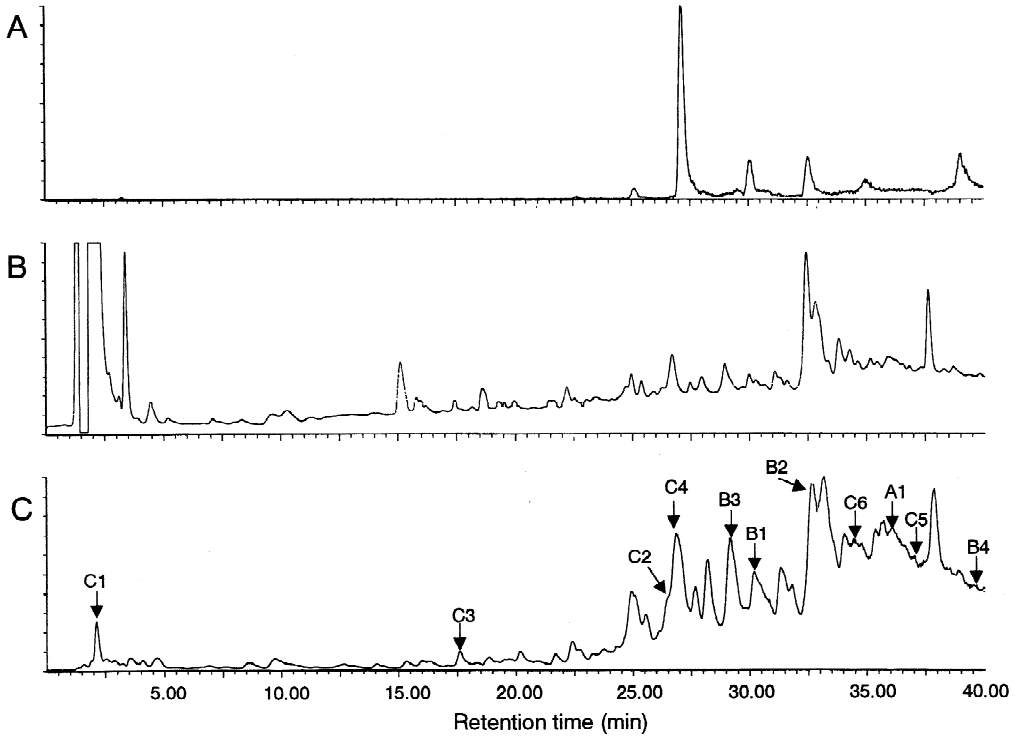


Fig. 1. Ultraviolet absorption, total ion and selected ion chromatograms of tryptic digest of native α-chymotrypsin. Tryptic digest of reduced/alkylated α-chymotrypsin (250 μg) was subjected to the LC–MS. (A) Selected ion chromatogram at $m/z$ 1265. (B) Ultraviolet absorption chromatogram at 215 nm. (C) Total ion chromatogram. The symbols in (C) correspond to the peptide fragments listed in Table 2.

Table 1
Primary structure of α-chymotrypsin (A, B and C chain)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| A-Chain | 1 | 11 | | | | | | |
| | C[#1]GVPAIQPVL | SGL | | | | | | |
| B-Chain | 1 | 11 | 21 | 31 | 41 | 51 | 61 | 71 |
| | IVNGEEAVPG | SWPWQVSLQD | KTGFHFC[#2]GGS | LINENWVVTA | AHC[#2]GVTTSDV | VVAGEFDQGS | SSEKIQKLKI | AKVFKNSKYN |
| | 81 | 91 | 101 | 111 | 121 | 131 | | |
| | SLTINNDITL | LKLSTAASFS | QTVSAVC[#1]LPS | ASDDFAAGTT | C[#3]VTTGWGLTR | Y | | |
| C-Chain | 1 | 11 | 21 | 31 | 41 | 51 | 61 | 71 |
| | ANTPDRLQQA | SLPLLSNTNC[#4] | KKYWGTKIKD | AMIC[#4]AGASGV | SSC[#5]MGDS*GGP | LVC[#3]KKNGAWT | LVGIVSWGSS | TC[#5]STSTPGVY |
| | 81 | 91 | | | | | | |
| | ARVTALVNWV | QQTLAAN | | | | | | |

[#1] to [#5] position of disulfide bonds.

* active site serine residue.

Underlined amino acids indicate the positions which are hydrolyzed by trypsin at the site of carboxylic terminal.

tified by the same LC–MS analysis, by comparing the observed mass numbers with the deduced ones (Table 2), except for the small fragments.

Fig. 3 shows the product ion spectrum of the $m/z$ 1265.0 ion as precursor ion. In Tables 3 and 4, the expected and obtained y-series and b-series fragment ions are listed. Although the N-terminal two amino acid residue could not be determined experimentally, the sequence of this peptide fragment was estimated to be DAMICAGASGVSSCMGDSGGPLVCK, using the ''MS-Digest'' and ''MS-Product'' software.

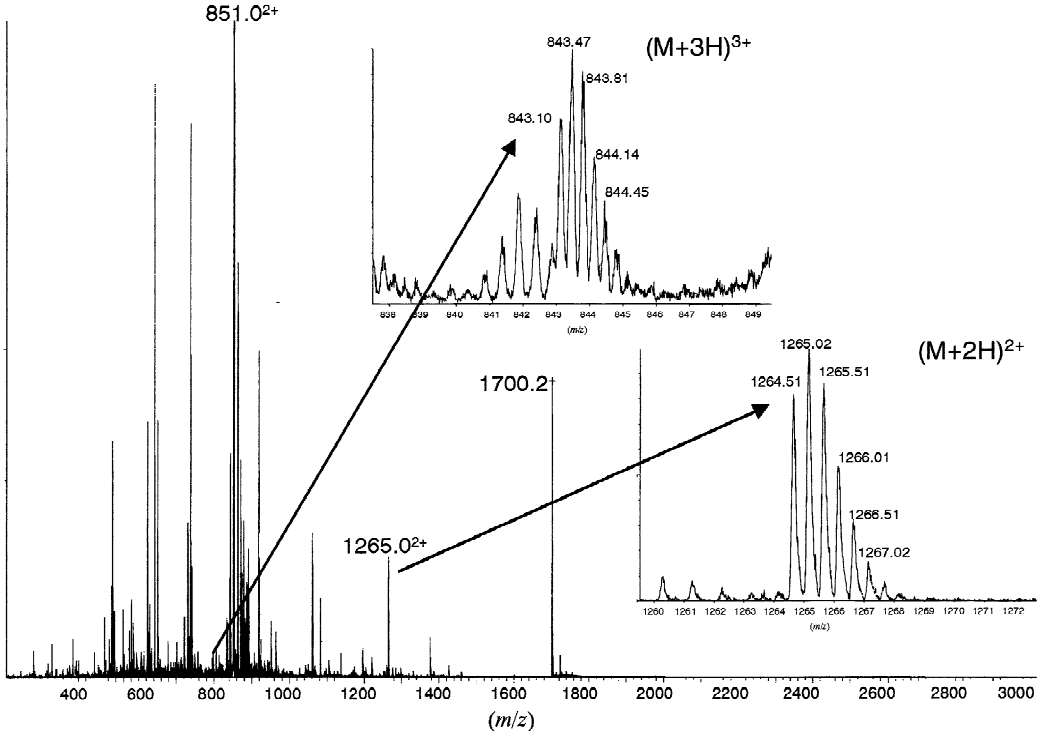


Fig. 2. Mass spectrum of peptide fragment (26.9 min, Fig. 1) containing active site serine residue obtained from native α-chymotrypsin.

Table 2
The primary structures and mass spectral data of deduced peptide fragments from native and DFP-inhibited α-chymotrypsins, as obtained by trypsin digestion

| No. | Chain | Start | End | Sequence | Obtained | Calculated | Calc. $(M+H)^+$ |
|---|---|---|---|---|---|---|---|
| A1 | A | 1 | 13 | CGVPAIQPVLSGL | $1324.8^{+}$ | $1324.7^{+}$ | $1324.7^{+}$ |
| B1 | B | 1 | 21 | IVNGEEAVPGSWPWQVSLQDK | $1170.6^{2+}$ | $1170.1^{2+}$ | $2339.2^{+}$ |
| B2 | B | 22 | 64 | TGFHFCGGSLINENWVVTAAHCGVTTSDVVVAGEFDQGSSSEK | $1147.1^{4+}$ | $1147.0^{4+}$ | $4585.1^{+}$ |
| B3 | B | 79 | 92 | YNSLTINNDITLLK | $811.1^{2+}$ | $811.4^{2+}$ | $1621.9^{+}$ |
| B4 | B | 93 | 130 | LSTAASFSQTVSAVCLPSASDDFAAGTTCVTTGWGLTR | $980.9^{4+}$ | $981.2^{4+}$ | $3921.9^{+}$ |
| C1 | C | 1 | 6 | ANTPDR | $673.0^{+}$ | $673.3^{+}$ | $673.3^{+}$ |
| C2 | C | 7 | 21 | LQQASLPLLSNTNCK | $850.8^{2+}$ | $851.0^{2+}$ | $1700.9^{+}$ |
| C3 | C | 23 | 27 | YWGTK | $654.1^{+}$ | $654.3^{+}$ | $654.3^{+}$ |
| C4 | C | 30 | 54 | DAMICAGASGVSSCMGDSGGPLVCK | $1265.1^{2+}$ | $1265.0^{2+}$ | $2529.1^{+}$ |
| C4* | C | 30 | 54 | DAMICAGASGVSSCMGD(S$^{\#}$)GGPLVCK | $1347.1^{2+}$ | $1347.1^{2+}$ | $2693.2^{+}$ |
| C5 | C | 56 | 82 | NGAWTLVGIVSWGSSTCSTSTPGVYAR | $943.6^{3+}$ | $943.5^{3+}$ | $2828.4^{+}$ |
| C6 | C | 83 | 97 | VTALVNWVQQTLAAN | $814.3^{2+}$ | $814.4^{2+}$ | $1627.9^{+}$ |

C4*: peptide containing phosphorylated serine residue.
$^{\#}$ Active center serine residue.

The sequence of the other peptide fragments listed in Table 2 were also identified by similar MS/MS measurement (data not shown).

### 3.2. DFP-inhibited α-chymotrypsin

Fig. 4 shows the UV absorbance, total and selected

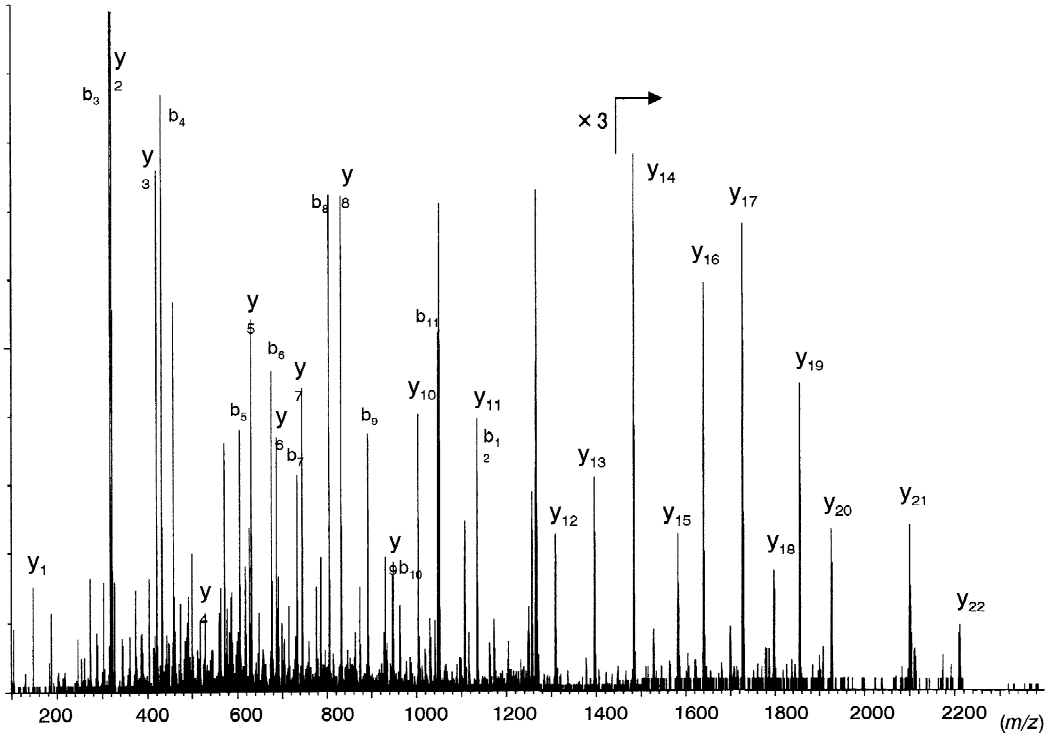


Fig. 3. Product ion mass spectrum of peptide fragment containing active site serine from native α-chymotrypsin. The $m/z$ 1265.0 ion in Fig. 2 was use as a precursor ion. All cysteine residues were alkylated with acrylamide.

Table 3
Comparison of observed y-series fragment ions with the deduced ones

| Sequence | Peptide | Native | | DFP adduct | | $\Delta^{\#}$ |
|---|---|---|---|---|---|---|
| | | Deduced | Observed | Deduced | Observed | |
| *C-terminal* | | | | | | |
| K | $y_1$ | 147.1 | 147.1 | 147.1 | 147.1 | 0.0 |
| C | $y_2$ | 321.2 | 321.1 | 321.2 | 321.1 | 0.0 |
| V | $y_3$ | 420.2 | 420.1 | 420.2 | 420.1 | 0.0 |
| L | $y_4$ | 533.3 | 533.3 | 533.3 | 533.1 | −0.1 |
| P | $y_5$ | 630.4 | 630.3 | 630.4 | 630.2 | 0.0 |
| G | $y_6$ | 687.4 | 687.3 | 687.4 | 687.2 | 0.0 |
| G | $y_7$ | 744.4 | 744.3 | 744.4 | 744.3 | 0.0 |
| S* | $y_8$ | 831.4 | 831.3 | 995.5 | 995.3 | 164.0 |
| D | $y_9$ | 946.5 | 946.3 | 1110.5 | 1110.5 | 164.2 |
| G | $y_{10}$ | 1003.5 | 1003.3 | 1167.6 | 1167.3 | 164.0 |
| M | $y_{11}$ | 1134.5 | 1134.3 | 1298.6 | 1298.4 | 164.1 |
| C | $y_{12}$ | 1308.6 | 1308.3 | 1472.6 | 1472.4 | 164.1 |
| S | $y_{13}$ | 1395.6 | 1395.4 | 1559.7 | 1559.4 | 164.1 |
| S | $y_{14}$ | 1482.6 | 1482.5 | 1646.7 | 1646.5 | 164.0 |
| V | $y_{15}$ | 1581.7 | 1581.4 | 1745.8 | 1745.6 | 164.2 |
| G | $y_{16}$ | 1638.7 | 1638.5 | 1802.8 | 1802.6 | 164.1 |
| S | $y_{17}$ | 1725.8 | 1725.6 | 1889.8 | 1889.7 | 164.1 |
| A | $y_{18}$ | 1796.8 | 1796.6 | 1960.9 | 1960.8 | 164.2 |
| G | $y_{19}$ | 1853.8 | 1853.7 | 2017.9 | 2017.7 | 164.1 |
| A | $y_{20}$ | 1924.9 | ND | 2088.9 | 2088.9 | – |
| C | $y_{21}$ | 2098.9 | ND | 2263.0 | 2263.0 | – |
| I | $y_{22}$ | 2212.0 | ND | 2376.1 | ND | – |
| M | $y_{23}$ | 2343.0 | ND | 2507.1 | ND | – |
| A | $y_{24}$ | 2414.1 | ND | 2578.1 | ND | – |
| D | $y_{25}$ | | | | | |
| *N-terminal* | | | | | | |

S* indicates the active center serine residue.
$\Delta^{\#}$ indicates the mass difference of fragment ions between DFP-inhibited and native α-chymotrypsins.
ND, not detected.

ion chromatograms of the tryptic digest of the DFP-inhibited α-chymotrypsin. On the extracted ion chromatogram for $m/z$ 1347.0 which corresponds to a doubly protonated peptide fragment containing the DFP-phosphorylated active site serine, one major peak was obtained, having a retention time of 32.3 min. In the mass spectrum of this peak (Fig. 5), doubly protonated ($m/z$ 1347.0) and triply protonated ($m/z$ 898.3) ions were obtained. On the extracted ion chromatogram of $m/z$ 1265.0, one peak also appeared, having a retention time of 26.9 min. This peak could be attributed to the peptide containing the active site serine, the same as that in the non-inhibited enzyme (Fig. 1). This suggests that the diisopropylphosphoryl moiety might have been partially removed from the active site serine residue during sample treatment, i.e. during reduction, alkylation and other processes, because the complete inhibition of α-chymotrypsin activity in the enzyme sample was verified by measurement of enzyme activity. The amount of the de-phosphorylated peptide peak estimated to be approximately one tenth that of the phosphorylated one, by comparing the areas of these two peaks on the respective selected ion chromatograms of $m/z$ 1265.0 and $m/z$ 1347.0.

The other peptide fragments derived from the DFP-inhibited α-chymotrypsin were also identified by the same LC–MS analysis, and were found to be identical to those derived from native α-chymotrypsin. This suggests that the DFP binding site is limited to only the active site serine residue. Fig. 6 shows the product ion spectrum of the $m/z$ 1347.0

Table 4
Comparison of observed b-series fragment ions with the deduced ones

| Sequence | Peptide | Native | | DFP adduct | |
|---|---|---|---|---|---|
| | | Deduced | Observed | Deduced | Observed |
| *N-terminal* | | | | | |
| D | $b_1$ | 116.0 | ND | 116.0 | ND |
| A | $b_2$ | 187.1 | ND | 187.1 | ND |
| M | $b_3$ | 318.1 | 318.0 | 318.1 | 318.0 |
| I | $b_4$ | 431.2 | 431.1 | 431.2 | 431.1 |
| C | $b_5$ | 605.2 | 605.1 | 605.2 | 605.1 |
| A | $b_6$ | 676.3 | 676.2 | 676.3 | 676.1 |
| G | $b_7$ | 733.3 | 733.2 | 733.3 | 733.2 |
| A | $b_8$ | 804.3 | 804.1 | 804.3 | 804.2 |
| S | $b_9$ | 891.4 | 891.2 | 891.4 | 891.2 |
| G | $b_{10}$ | 948.4 | 948.2 | 948.4 | 948.2 |
| V | $b_{11}$ | 1047.5 | 1047.3 | 1047.5 | 1047.3 |
| S | $b_{12}$ | 1134.5 | 1134.3 | 1134.5 | 1134.3 |
| S | $b_{13}$ | 1221.5 | 1221.4 | 1221.5 | 1221.5 |
| C | $b_{14}$ | 1395.6 | 1395.4 | 1395.6 | 1395.3 |
| M | $b_{15}$ | 1526.6 | 1526.4 | 1526.6 | 1526.3 |
| G | $b_{16}$ | 1583.6 | ND | 1583.6 | 1583.6 |
| D | $b_{17}$ | 1698.7 | ND | 1698.7 | ND |
| S* | $b_{18}$ | 1949.8 | ND | 2113.9 | ND |
| G | $b_{19}$ | 2006.8 | ND | 2170.9 | ND |
| G | $b_{20}$ | 2063.8 | ND | 2227.9 | ND |
| P | $b_{21}$ | 2160.9 | ND | 2325.0 | ND |
| L | $b_{22}$ | 2273.9 | ND | 2438.0 | ND |
| V | $b_{23}$ | 2373.0 | ND | 2537.1 | ND |
| C | $b_{24}$ | 2547.1 | ND | 2711.2 | ND |
| K | $b_{25}$ | | | | |
| *C-terminal* | | | | | |

S* indicates the active center serine residue.
ND, not detected.

peak ion as a precursor ion. The expected and observed y-series and b-series fragments are listed in Tables 3 and 4, respectively. The fragment ions from $y_1$ to $y_7$ were identical for both the native and DFP-inhibited enzymes (see Figs. 3 and 6). On the other hand, the fragment ions from $y_8$ to $y_{24}$ in the DFP-inhibited peptide showed a +164 Da mass shift compared to those in the native enzyme. This mass difference is assumed to be derived from the mass difference between serine and diisopropylphosphoryl–serine. The sequence of this peptide fragment was determined to be DAMICAGASGVSSCMGDS*GGPLVCK (S*: phosphorylated serine), although the N-terminal two amino acid residues could not be determined experimentally.

The sequence of the other peptide fragments of the DFP-inhibited α-chymotrypsin were also identified in the similar MS/MS analysis.

## 4. Discussion

Using commercially available purified α-chymotrypsin, we report on an analytical method for the detection of the DFP-binding peptide fragment in the tryptic digest of α-chymotrypsin by LC–MS, and for the structural confirmation of the peptide as well, by the determination of the amino acid sequence using LC–MS/MS. We were able to ascertain a +164 Da mass shift of the active site serine residue of the DFP inhibited enzyme compared to the native enzyme.

Nagao et al. [15] confirmed that Sarin was bound

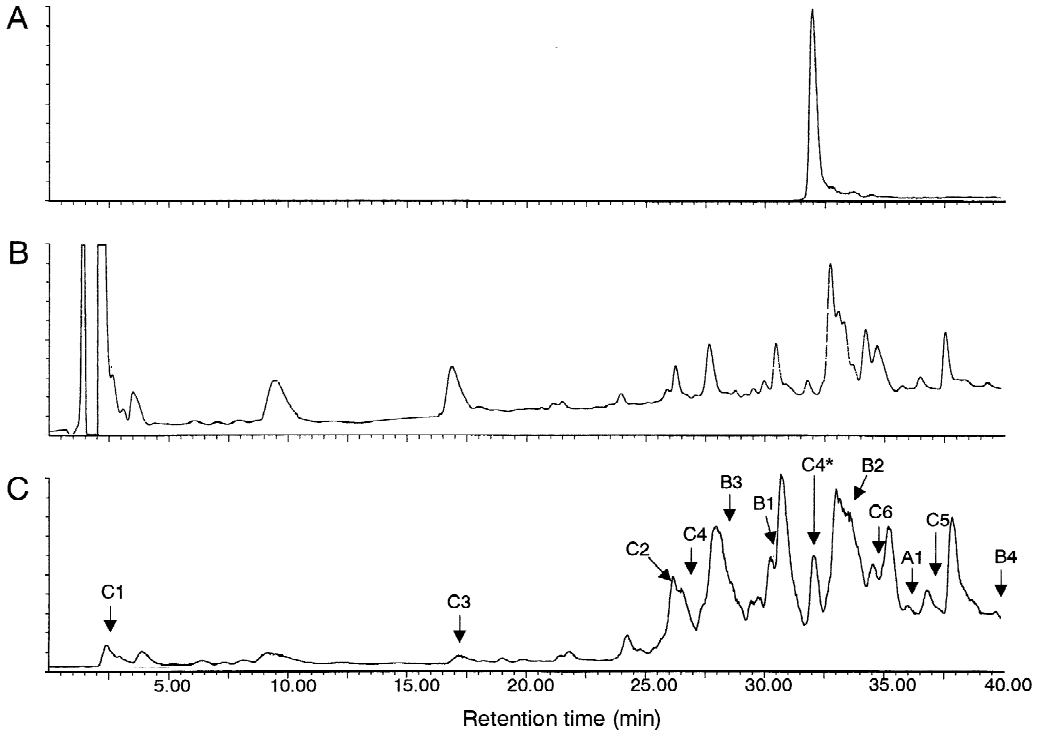


Fig. 4. Ultraviolet absorption, total ion and selected ion chromatograms of tryptic digest of DFP-inhibited α-chymotrypsin. Tryptic digest of reduced/alkylated α-chymotrypsin inhibited by DFP (250 μg) was subjected to the LC–MS. (A) Selected ion chromatogram at $m/z$ 1347. (B) Ultraviolet absorption chromatogram at 215 nm. (C) Total ion chromatogram. The symbols in (C) are corresponding to the peptide fragments listed in Table 2.

to the erythrocyte acetylcholinesterases obtained from victims who were poisoned in the Tokyo Subway Sarin gas attack. This was accomplished by the use of immunoaffinity chromatography, phosphatase treatment, trimethylsilylation and gas chromatography–mass spectrometric (GC–MS) analysis. Polhuijs et al. [16] detected Sarin from a serum sample of a victim of the Matsumoto Sarin gas attack by GC–MS after the addition of fluoride ion. In addition, Barak et al. [17] demonstrated a method for the direct detection of Sarin and Soman adducts of human recombinant acetylcholinesterases. Noort et al. established a retrospective method for confirming mustard gas [18] and phosgene [19] exposure by the detection of adducts of human hemoglobin and albumin using LC–MS analysis after proteolytic digestion. Black et al. also reported the analytical method of detection the alkylation products of human hemoglobin with sulfur mustard by LC–MS [20].

The mass spectrometric strategy provides direct evidence for the inhibition of serine hydrolase by irreversible inhibitors. Using the technique of tryptic digestion, in conjuction with LC–MS/MS, we also confirmed the DFP-binding to trypsin (EC. 3.4.21.1) and the binding of Sarin to α-chymotrypsin and trypsin (data not shown). The direct confirmation of binding of the organophosphorus compounds to the target enzyme (acetylcholinesterase and butyrylcholinesterase; EC 3.1.1.8) by the mass spectrometric determination of enzyme adducts seems crucial in the elucidation of exposure and poisoning by organophosphorus compounds. We are now in the process of developing an LC–MS/MS technique for the elucidation of adduct formation of cholinesterases with organophosphorus compounds.

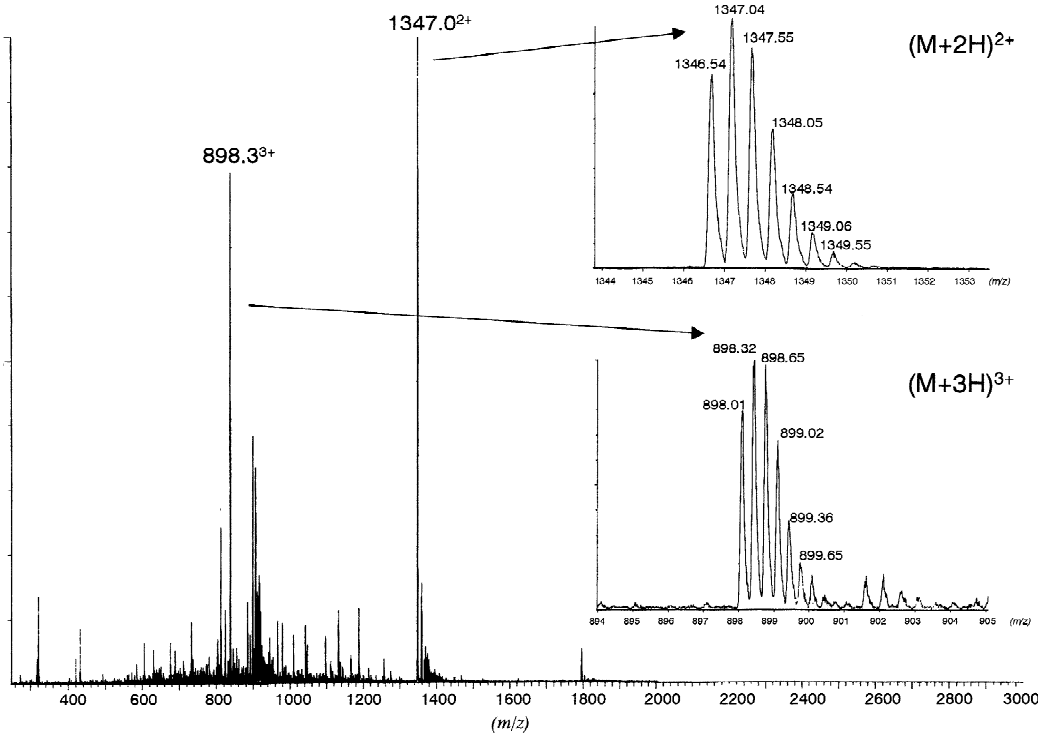


Fig. 5. Mass spectrum of peptide fragment containing active site serine (32.3 min, Fig. 4) of DFP-inhibited α-chymotrypsin.

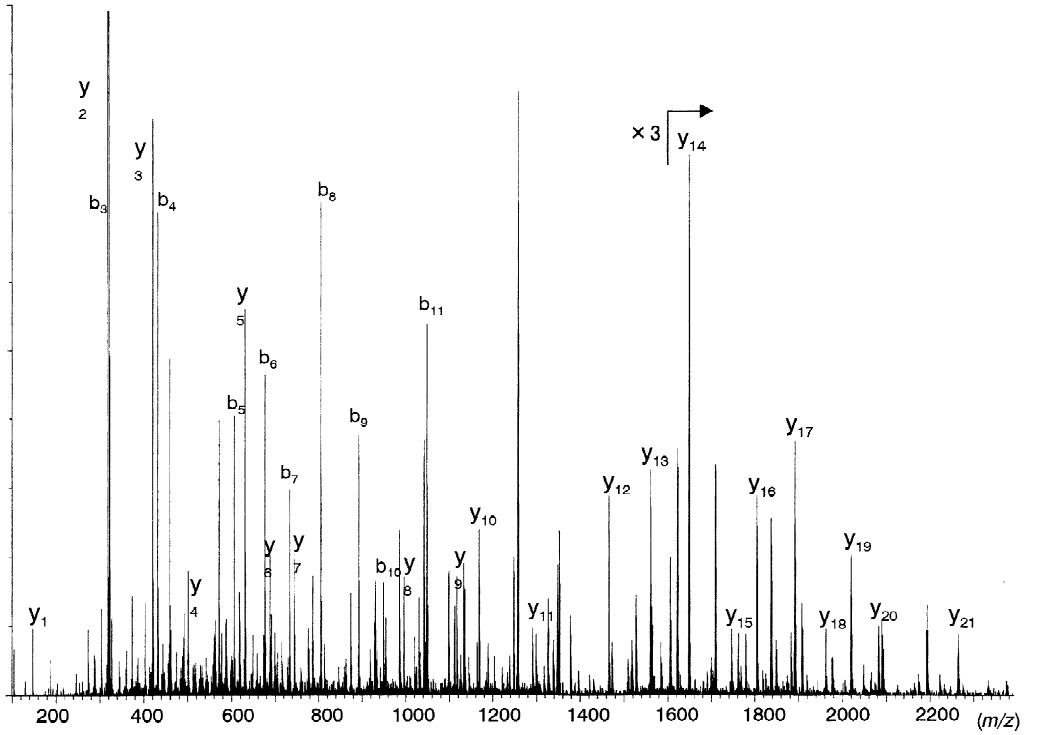


Fig. 6. Product ion mass spectrum of active site serine containing peptide fragment of DFP-inhibited α-chymotrypsin. The $m/z$ 1347 ion was use as a precursor ion (32.3 min, Fig. 4). All cysteine residues are alkylated by acrylamide.

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