A Comprehensive Analysis of Selenium-Binding Proteins in the Brain Using Its Reactive Metabolite

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The intracellular metabolism of selenium in the brain currently remains unknown, although the antioxidative activity of this element is widely acknowledged to be important in maintaining brain functions. In this study, a comprehensive method for identifying the selenium-binding proteins using PenSSeSPen as a model of the selenium metabolite, selenotrisulfide (RSSeSR, STS), was applied to a complex cell lysate generated from the rat brain. Most of the selenium from L-penicillamine selenotrisulfide (PenSSeSPen) was captured by the cytosolic protein thiols in the form of STS through the thiol-exchange reaction (R-SH+PenSSeSPen→RSSeSPen+PenSH). The cytosolic protein species, which reacted with the PenSSeSPen mainly had a molecular mass of less than 20kDa. A thiol-containing protein at m/z 15155 in the brain cell lysate was identified as the cystatin-12 precursor (CST12) from a rat protein database search and a tryptic fragmentation experiment. CST12 belongs to the cysteine proteinase inhibitors of the cystatin superfamily that are of interest in mechanisms regulating the protein turnover and polypeptide production in the central nervous system and other tissues. Consequently, CST12 is suggested to be one of the cytosolic proteins responsible for the selenium metabolism in the brain.

Key words selenium; selenotrisulfide; cystatin-12 precursor; mass spectrometry; thiol-exchange

Selenium is an essential micronutrient for humans and other mammals that require its biological activity.3 After the intake of a variety of selenium compounds in one’s diet, this element genetically occurs as the unique amino acid selenocysteine (SeCys, Sec or U) in proteins, called selenoproteins.2 Selenoproteins are ubiquitously expressed in all organs and tissues. Selenium-dependent glutathione peroxidases (GPx-1, GPx-2, GPx-3, GPx-4 and GPx-6) can catalyze the reduction of certain peroxide species (R-OOH) to alcohols (R-OH) at their active center SeCys residue.3 Of all these GPxs, GPx-4 is the only antioxidative enzyme that can directly reduce phospholipid hydroperoxides generated in biological membranes.

Although the brain claims slightly ca. 2% of the body mass in humans, it is responsible for approximately 20% of total body oxygen consumption.1–6 As a consequence of the high oxygen demand, the brain tissue inevitably induces the generation of large amounts of reactive oxygen species, which are thought to be associated with the onset and/or progression of neurodegenerative diseases such as Alzheimer’s disease2–9 and Parkinson’s disease,10–12 due to the reactive oxygen species-mediated injury from the early stages of the illnesses. Numerous papers have pointed out that decreases in the GPx activity in the brain are associated with these neurodegenerative diseases.13–15 In particular, the role of GPx-4 appears important due to the high lipid content in the brain. Its activity in neurons is likely to be more important than in the other cells in the brain, because nearly 80% of the energy in the brain generated by respiration is consumed to support ongoing neuronal signaling.16

The selenium concentration in the brain is reported to be far less variable than those in the peripheral tissues and organs in laboratory rodents. This fact implied the importance of selenium for maintaining the integrity of brain functions, and the distinctive selenium metabolism in the brain. It also raised a question of whether such homeostatically maintained selenium storage in the brain results from the neuron-specific metabolism and retention, the regulatory mechanism of the blood–brain barrier or both of them.17,18 Currently, the intracellular reduction and/or transport (metabolism) of selenium in the brain still remain unknown, because of a) trace elements, b) diversity of oxidation state accompanying the metabolic reduction, and c) few effective probe materials responsible for the selenium metabolic species.

Certain reduced forms of selenium are thought to be reactive with various endogenous molecules, particularly thiol-containing proteins, and the resulting selenium-protein complexes may participate in its subsequent metabolism and/or brain-specific retention. Cysteine (Cys) is the most redox active of the commonly encoded amino acids and is thus an important target responsible for the selenium atom. Selenotrisulfide (RSSeSR, STS), one of the reduced forms, was actually detected in a biological system, i.e., a selenium-enriched yeast sample using modern mass spectrometric techniques,19 and its reactivity with biogenic protein thiols has been studied.20–24 In our previous study, we investigated the reactivity of rat liver cytosolic proteins with STS as one of the reactive metabolic intermediates.25 Several cytosolic proteins with Cys thiol were found to be reactive with a selenotrisulfide derivative through the thiol-exchange reaction. The most distinctive thiol-containing protein in the cell lysate was successfully identified as the rat liver fatty acid-binding protein (LFABP). When selenious acid was intraperitoneally administered to mice, a 14-kDa mouse liver selenium-binding protein that has only the free thiol at Cys69 was identified using a combination of the radioactive tracer (H75SeO3) and Western blotting techniques.26 When our methodology was applied...
to the mouse liver cell lysate, we verified that the selenium from l-penicillamine selenotrisulfide (PenSSeSPen) was actually bound to the mouse LFABP in the same reaction manner through only its Cys69, as was observed for the rat LFABP. In the present study, we extended this methodology to search for the selenium-binding proteins in the brain.

**Experimental**

**Materials** Selenious acid, trifluoroacetic acid, N-ethylmaleimide (NEM) and trypsin (from porcine pancreas, 1000–2000 units mg\(^{-1}\), salt free) were purchased from Nacalai Tesque, Inc. l-Penicillamine (Pen), glutathione in the reduced form (GSH) and 2,3-diaminonaphthalene (DAN) were obtained from Tokyo Chemical Industry Co., Ltd. Bovine serum albumin (BSA) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were from the Sigma Co. The dialysis tube Spectra/Por Membrane (regenerated cellulose, molecular weight cutoff; 6–8 kDa) was from Spectrum Laboratories, Inc. Matrix-assisted laser desorption ionization time-of-flight-mass spectrometry (MALDI TOF-MS)–grade sinapinic acid was also from the Sigma Co. The atomic absorption spectrometry-grade selenium standard solution [1000 ppm as selenium(IV) dioxide in 0.5 M nitric acid] was from Kanto Chemical Co., Inc. The water used (\(>18 \text{ MΩ} \cdot \text{cm}\)) was generated by a Milli-Q Biocel system (Millipore Corp.). All other chemicals were of commercial reagent or special grade and used as received.

**Synthesis of PenSSeSPen** l-Penicillamine selenotrisulfide (PenSSeSPen, Fig. 1 top) was synthesized according to a previously described procedure.\(^{27,28}\) Briefly, a 1 mM selenious acid solution was poured into a 4 mM Pen solution and the mixture was allowed to react with stirring for 3 h at room temperature and left for another 20 h at 4°C. The resulting snow-white precipitate was isolated and carefully washed twice with cold water and then washed twice with cold methanol. The obtained material was dried in vacuo at room temperature for 24 h or longer, then stored in a desiccator until used. PenSSeSPen was dissolved in Milli-Q water just before use. An equal volume of a 1 mM DTNB solution in a 10 mM Tris–HCl buffer (pH 7.4) was added to the cell lysates samples. After incubation for 30 min, the absorbance at 450 nm was monitored by a V-660 spectrophotometer (JASCO Corp.). GSH was used as the standard compound for preparation of the calibration curve.

**Preparation of Rat Brain Cell Lysate and Reaction with PenSSeSPen** A male Wister rat (3–4 weeks old, body weight ≈120 g) was sacrificed under ether anesthesia and its brain was removed. All experiments with live animals were performed in compliance with the guidelines of the Nagasaki University on Animal Care and Use, and the institutional committee has approved the experimental protocols. The isolated brain was thoroughly rinsed with 10 mM Tris–HCl buffer (pH 7.4). The brain tissue was homogenized by a Polytron PT1200E (Kinematica AG) and then sonicated at acoustic power levels of 20 W by a probe-type sonicator 250D (Branson Ultrasonic Corp.). The brain tissue suspension was transferred to a polycarbonate centrifuge tube (38×102 mm). Subsequently, the brain cell lysate was obtained by centrifugation in a rotor TYPE45Ti on a L-80 ultracentrifuge (Beckman Coulter Inc.) at 20000 rpm for 60 min at 4°C. The obtained supernatant was used in the experiments. The protein concentrations in the resulting cell lysate were determined by the Lowry method using bovine serum albumin as the standard material.

PenSSeSPen solutions at desired concentrations were dissolved in Milli-Q water just before mixing with the brain cell lysate. The PenSSeSPen solutions (0.4 mL) were combined with the rat brain cell lysate (3.6 mL), and then the mixture was allowed to react for 30 min at 37°C. The NEM solution in Milli-Q water at 10 mM (0.4 mL) was also allowed to react with the brain cell lysate (3.6 mL) for 10 min at 37°C.

**X-Ray Photoelectron Spectroscopy (XPS)** The XPS determination of selenium and protein thiol concentrations in the resulting cell lysate was determined by the Lowry method using bovine serum albumin as the standard material.

An equal volume of a 1 mM DTNB solution in a 10 mM Tris–HCl buffer (pH 7.4) was added to the cell lysates samples. After incubation for 30 min, the absorbance at 450 nm was monitored by a V-660 spectrophotometer (JASCO Corp.). GSH was used as the standard compound for preparation of the calibration curve.
analysis for this study was conducted using a Shimadzu/ Kratos AXIS-ULTRA instrument fitted with a delay-line detector, and a monochromated aluminum $K_{\alpha}$ line (wavelength: 0.8339nm, 1.486keV) operating at 10kV and 13mA. The samples were analyzed at a pressure of 5x10^{-10} Torr and room temperature. Freeze-dried brain cell lysate samples were deposited on a conducting carbon tape and mounted on a stainless steel bar prior to sample loading in the spectrometer for the XPS analysis. All surfaces were examined in the survey mode over the binding energy range from 0 to 1100 eV in order to identify all species. The high-resolution scans of carbon 1s were performed over the range of 280–290 eV with a pass energy of 40 eV and step size of 0.05 eV. The XPS spectra were collected in the binding energy form and fit using a nonlinear least-square curve fitting program (XPSPEAK41 Software).

The spectra presented include those of the selenium 3p and sulfur 2p photoemission peaks, which can be deconvoluted using a peak fitting procedure. The selenium 3p and sulfur 2p peaks are doublets as a result of spin orbit splitting; the selenium 3p 1/2 and sulfur 2p peaks are, respectively, the stronger peaks with the selenium 3p 1/2 and sulfur 2p 1/2 being half their intensity. Peaks fitted to the selenium 3p and sulfur 2p spectra use a summed Gaussian–Lorentzian (SGL) function to fit the individual peak components. The Shirley background correction method was used to allow accurate fitting of the peak components.\(^{25}\)

MALDI TOF-MS The sample solutions were mixed with an equal volume of matrix solution [saturated sinapinic acid in 0.1% trifluoroacetic acid and 34% acetonitrile for the specimens], and an aliquot was applied on an AnchorChip target (Bruker Daltonics, Inc.) that was loaded with a sinapinic acid matrix thin layer. The mass spectra were acquired in the linear positive ion mode using an Ultraflex MALDI TOF/TOF-MS (Bruker Daltonics, Inc.). Each spectrum was produced by accumulating data from 5000 consecutive laser shots. The molecular mass calibration was carried out using the \#206355 Protein Calibration Standard. Both the PenSSeSPen- and NEM-reactive protein species were subjected to a database search using the Protein Information Resource (http://www-nbrf.georgetown.edu/pirwww/). The candidate proteins were identified by the tryptic fragment mass data from the MS-Digest program of Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm) (Table S1). The brain cell lysate samples in the molecular mass range of interest were separated by ultrafiltration through a regenerated cellulose membrane (molecular mass cutoff: 30kDa) and then dialyzed using a regenerated cellulose membrane of molecular mass cutoff 6–8kDa before the tryptic digestion.

Results and Discussion

In general, the selenotrisulfide (STS) species formed with low molecular mass thiols [e.g., cysteine (Cys) and glutathione (GSH)] are chemically labile under physiological conditions (Fig. S1, ESF). We synthesized an STS compound using L-penicillamine (Pen) as a thiol instead of Cys and GSH. The Pen molecule has two methyl groups at the $\beta$ carbon atom of Cys. Pen is structurally similar to Cys, but is capable of generating a chemically stable STS species [L-penicillamine selenotrisulfide (PenSSeSPen), Fig. 1 top]. This compound can be easily isolated from the reaction mixture and is distinctly stable without any degradation for over 24 h or longer under physiological conditions.\(^{27}\) The elemental analysis results of PenSSeSPen used in this study were in good agreement with the calculated values. When the PenSSeSPen sample was subjected to a MALDI TOF-MS analysis, the distinctive selenium isotopic pattern involving one selenium atom in a molecular ion was detected and the $^{79}$Se-containing molecular ion peak was at $m/z$ 376.3 (Calcd for $^{79}$Se 376.2). PenSSeSPen can react with various biogenic thiols through the thiol-exchange reaction\(^{27,33,34}\) (Fig. 1). Cys thiols are weak acids, but the thiol microenvironment in proteins can influence its $pK_{a}$ value. The reactivity of thiols is closely related to its $pK_{a}$ value.\(^{37}\) GSH, the most abundant low mass thiol in the cell cytosol, has a $pK_{a}$ of 9.1.\(^{38}\) The Cys thiol becomes a stronger nucleophile and readily reacts with electrophilic species in biological systems. With its remarkable reactivity, the Cys thiol could possibly play critical roles in the selenium reduction and/or transport. Such a remarkable nucleophilicity renders Cys the most common target for the selective selenium conjugation.\(^{39}\) The N-ethylmaleimide (NEM)-reactive protein thiols did not necessarily allow the reaction with PenSSeSPen, which seems to result from the chemical diversity of the protein thiols in the brain cell lysate.

To examine the reactivity of STS with cytosolic protein species, the rat brain cell lysate was incubated with PenSSeSPen for 30 min at 37°C, and then the protein-bound selenium and protein thiol concentrations in the resulting cell lysate were determined by the 2,3-diaminonaphthalene (DAN) method subsequent to the acid digestion and the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) method, respectively (Table 1). In most cases, thiol-containing peptides and proteins are associated with the selenium metabolism and/or interactions of resulting metabolic species.\(^{27,33}\) The protein thiol concentration in the brain cell lysate decreased with the increasing PenSSeSPen concentration, meanwhile, the protein-bound selenium concentration inversely increased (Table 1). Thus, selenium from PenSSeSPen was captured by the protein species in the brain cell lysate as a consequence of the reaction of PenSSeSPen with their reactive thiols.

Subsequently, to obtain information on the oxidation state and chemical bond of selenium and sulfur atoms, the brain cell lysate samples were analyzed by X-ray photoelectron spectroscopy (XPS). Selenium 3p and sulfur 2p electrons from the brain cell lysate before the reaction with PenSSeSPen [Fig. 2(A)] gave an absorption peak at 163–166 eV. On the other hand, the selenium 3p and sulfur 2p electrons in the brain cell

<table>
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<th>Table 1. Changes in Selenium and Protein Thiol Contents in the Brain Cell Lysate after Reaction with PenSSeSPen$^a$</th>
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<tbody>
<tr>
<td>PenSSeSPen concn in the reaction mixture (nmol)</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.001</td>
</tr>
<tr>
<td>0.01</td>
</tr>
<tr>
<td>0.1</td>
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$^a$ Prepared brain cell lysate (37.3 nmol) and PenSSeSPen solution was mixed in the ratio of 1:9 by volume to make final selenium concentration 0.001 to 0.1 nmol. Reaction time: 30 min, Reaction temperature: 37°C. $^b$ Values are the mean±standard error ($n=4$).
lysate after the reaction with PenSSeSPen [Fig. 2(B)] provided two distinctive absorption peaks at 162–166 and 167–172 eV. Both absorption peaks from the brain cell lysate after the reaction with PenSSeSPen were separated into the selenium 3p and sulfur 2p components. The peak envelop of the selenium 3p and sulfur 2p components from the brain cell lysate after the reaction with PenSSeSPen (black broad dotted lines in Fig. 2) was consistent with the observed spectrum (black broad solid lines in Fig. 2). The absorption peak shape of the brain cell lysate after the reaction with PenSSeSPen [Fig. 2(B)] was almost similar to that of PenSSeSPen [Fig. 2(C)]. In addition, the binding energy of the selenium 3p and sulfur 2p components of the brain cell lysate was also nearly identical to those of the PenSSeSPen (Table 2). Therefore, selenium bound to the protein species in the brain cell lysate was thought to mostly exist in the STS form.

The brain cell lysate after the reaction with PenSSeSPen was subjected to a MALDI TOF-MS analysis. The mass spectral peaks of the thiol-containing proteins in the brain cell lysate were specified by the molecular mass gain in m/z by 125 after chemical derivatization (alkylation) with NEM in comparison to that before the reaction. Protein thiol alkylation with NEM is faster than other low molecular mass thiol-modifying reagents, resulting in the selective reaction toward the thiols at physiological pH. \(^{10,43}\) Under the employed reaction conditions, the protein thiol content after the reaction with NEM decreased to 4.38±2.29% \((n=4)\) of that before the reaction. Thus, the reactive protein thiols appeared to be mostly probed by the NEM alkylation. The distinctive NEM-reactive mass spectral peaks from the brain cell lysate were observed in the range of molecular mass less than 20 kDa (Fig. S2, ESI†). As was listed in Table 3, twelve kinds of proteins with the molecular masses of 2000 to 20000 in m/z allowed a reaction with NEM, and three species of them resulted in the molecular mass gain in m/z by 226 after the reaction with PenSSeSPen. Such an increase in the molecular mass due to the reaction with PenSSeSPen corresponded to that of the selenenyl-penicillamine (SeSPen) moiety, indicating that PenSSeSPen could possibly react with the three protein species through a thiol-exchange reaction to form the asymmetrical STS \((R\text{-Cys}-SSeSPen\rightarrow R\text{-Cys-SSeSPen}+\text{PenSH})\). The NEM-reactive protein thiols did not necessarily allow the reaction with PenSSeSPen, which seems to result from the chemical diversity of the protein thiols in the cell lysate.

The three PenSSeSPen-reactive protein species were subjected to a database search using the Protein Information Resource \((http://www-nbrf.georgetown.edu/pirww/)\). The protein species of m/z 15155.5 and 15196.5 were predicted as the brain cystatin-12 precursor \((\text{CST12, amino acid length 128, calculated molecular mass 15152.4)}\) and hemoglobin \(\alpha\)-chain, respectively. No appropriate protein species was found for the molecular ion peak at m/z 12352.9. Shown in Fig. 3 are representative mass spectra of the m/z 15155.5 protein species \([\text{Fig. 3(A)}]\), its NEM-adduct \([\text{Fig. 3(B)}]\) and its SeSPen-adduct \([\text{Fig. 3(C)}]\). CST12 is a secretory protein that is composed of the signal peptide \((\text{Met1–Phe21)}\) and the main chain \((\text{Lys22–Thr128)}\). CST12 also involves five Cys residues; Cys19 is the only free thiol and two intramolecular disulfide bonds are formed between Cys 82 and Cys 92 and between Cys 105 and Cys 125 \((\text{Fig. 4)}\). PenSSeSPen was thought to react with the Cys19 thiol of CST12. The brain cell lysate samples in the molecular mass range of interest were separated by ultrafiltration \((\text{Fig. S3, ESI)}\) and then dialyzed using a regenerated cellulose membrane of molecular mass cutoff 6–8 kDa. The obtained cell lysate samples were subjected to MALDI TOF-MS sub-

<table>
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<tr>
<th>Atom level</th>
<th>Brain cell lysate after reaction with PenSSeSPen</th>
<th>PenSSeSPen</th>
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<tbody>
<tr>
<td>Se 3p(^{3/2})</td>
<td>163.30</td>
<td>162.05</td>
</tr>
<tr>
<td>Se 3p(^{1/2})</td>
<td>168.04</td>
<td>167.61</td>
</tr>
<tr>
<td>S 2p(^{3/2})</td>
<td>163.12</td>
<td>162.93</td>
</tr>
<tr>
<td>S 2p(^{1/2})</td>
<td>164.49</td>
<td>164.20</td>
</tr>
</tbody>
</table>

Fig. 2. X-Ray Photoelectron Spectroscopy of Selenium 3p and Sulfur 2p Electrons

(A) Brain cell lysate before reaction with PenSSeSPen, (B) brain cell lysate after reaction with 0.1 mM PenSSeSPen for 30 min, (C) PenSSeSPen. Black broad solid line: found, Black narrow dotted line: calcld Se 3p\(^{3/2}\), Black narrow solid line: calcld Se 3p\(^{1/2}\), Gray narrow dotted line: calcld S 2p\(^{3/2}\), Gray narrow solid line: calcld S 2p\(^{1/2}\). Black broad dotted line: envelope calcld. Ground state electronic configuration of selenium and sulfur atoms: \([\text{Ne}] 3s^2 3p^6 3d^{10} 4s^2 4p^4 \) and \([\text{He}] 2s^2 2p^6 \).
sequent to the trypsin digestion. Characteristic tryptic fragments from CST12 (Met1–Lys22, Ser5–Lys28, Asn29–Lys55, Asn29–Arg58, Thr80–Lys101, Cys105–Thr128) were detected from the brain cell lysate after the tryptic digestion (Fig. 5). On the other hand, the molecular ion peak at \( m/z \) 15155.5 had disappeared from the mass spectrum of the trypsin-digested cell lysate. Taking all these results together, the protein species of \( m/z \) 15155.5 was identified as the rat CST12. This protein is a member of the Cys proteinase inhibitors (the cystatin superfamily) that are of interest to mechanisms regulating protein turnover and polypeptide production in the central nervous system and other tissues. The amino acid sequence of CST12 is also conserved among the mouse, rat, bovine and human species. The cystatins, as well as the selenium element, are known to widely distribute in all body tissues. Particularly, their physiological roles in tumorigenesis and neurodegenerative diseases should be given increased attention.

**Conclusion**

In conclusion, a profiling method for identifying the selenium-binding proteins using PenSSeSPen as a model of the selenium metabolite STS was applied to the complex cell lysate generated from the rat brain. The XPS analysis demonstrated that selenium from PenSSeSPen was mostly bound to the cytosolic protein thiols in the form of STS through the thiol-exchange reaction. The subsequent MALDI TOF-MS analysis indicated that the cytosolic protein species with molecular mass less than 20kDa were mainly reactive with PenSSeSPen. A Cys-containing protein at \( m/z \) 15155.5 in the brain cell lysate was identified as CST12 from a rat protein database search and a tryptic fragmentation experiment. Overall, CST12 was suggested as one of the cytosolic proteins responsible for the selenium metabolism in the brain by a comprehensive analysis using a STS species. Taking the physiological roles of cystatins into consideration, the nature of the antioxidant selenium physiological linkage to the rat CST12 needs to be explored. This methodology using a combination of the MS technique and a reactive selenium metabolite could be effective for obtaining potential clues about the selenium-binding proteins and/or selenium-interactive species in biological systems. Consequently, it will lead to a better understanding of the selenium metabolism and functions in mammals that are required for this element as an essential micronutrient.

**Table 3. Observed Molecular Mass Gain in MALDI TOF-MS of the Rat Brain Cell Lysate before and after the Reaction with NEM and PenSSeSPen**

<table>
<thead>
<tr>
<th>Original peak in ( m/z ) before reaction (a)</th>
<th>Peak in ( m/z ) after reaction with NEM (a+125, +250 and +375)</th>
<th>Peak in ( m/z ) after reaction with PenSSeSPen (a+226)</th>
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<tbody>
<tr>
<td>4406.0</td>
<td>4532.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>4562.6</td>
<td>4688.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>7534.9</td>
<td>7660.6, 7786.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>7924.5</td>
<td>8050.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>11253.7</td>
<td>11361.2, 11487.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>11790.9</td>
<td>11917.5, 12042.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>13235.9</td>
<td>12476.9, 12602.0, 12727.0</td>
<td>12576.5</td>
</tr>
<tr>
<td>15155.5</td>
<td>15282.8</td>
<td>15380.1</td>
</tr>
<tr>
<td>15196.5</td>
<td>15322.7, 15447.8</td>
<td>15422.5</td>
</tr>
<tr>
<td>15847.0</td>
<td>16098.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>17741.7</td>
<td>17867.6, 17991.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>17791.2</td>
<td>17914.0, 18040.6</td>
<td>n.d.</td>
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(a) NEM and PenSSeSPen concentrations used: 1 and 0.1 mM. Reaction time: 10 and 30 min. Reaction temperature: 37°C. n.d.: not detected.

Fig. 3. MALDI TOF-MS of the PenSSeSPen Reactivity to \( m/z \) 15155.5 Species in the Rat Brain Cell Lysate

(A) Before reaction, (B) after reaction with 1 mM NEM for 10 min, (C) after reaction with 0.1 mM PenSSeSPen for 30 min. Reaction temperature: 37°C. Arrows indicate the peaks at \( m/z \) 15155.5, 15282.8 (NEM-adduct) and 15380.1 (SeSPen-adduct).
Fig. 4. Primary Structure of the Rat Cystatin-12 Precursor

This protein is composed of the signal peptide (Met1–Phe21) and the main chain (Lys22–Thr128). Two intramolecular disulfide bonds between Cys82 and Cys92 and between Cys105 and Cys125. Cys19 has only free thiol.

Fig. 5. MALDI TOF-MS of Trypsin-Digested Rat Cystatin-12 Precursor Fragments in the Rat Brain Cell Lysate

(A) Met1–Lys22 (molecular mass calcld for Met acetyl 2513.1, found 2517.7), (B) Ser5–Lys28 (calcld 2656.5, found 2646.6), (C) Asn29–Lys55 (calcld 3335.4, found 3331.0), (D) Asn29–Arg58 (calcld 3754.1, found 3755.6), (E) Thr80–Lys101 (calcld 2424.1, found 2423.7), (F) Cys105–Thr128 (calcld 2806.4, found 2801.6). Asterisks indicate the fragment peaks.
Conflict of Interest  The authors declare no conflict of interest.

Supplementary Materials  The online version of this article contains supplementary materials.

References