Anal. Chem. **2004,** *76,* 728−735

Fluorogenic Derivatization Reagents Suitable for Isolation and Identification of Cysteine-Containing Proteins Utilizing High-Performance Liquid Chromatography−Tandem Mass Spectrometry

Mayumi Masuda,[†] Chifuyu Toriumi,[†] Tomofumi Santa,[†] and Kazuhiro Imai*[,‡]

Laboratory of Bio-Analytical Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and Research Institute of Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi, Nishitokyo-shi, Tokyo 200-8585, Japan

The fluorogenic derivatization reagents with a positive charge, 4-(dimethylaminoethylaminosulfonyl)-7-chloro-2,1,3-benzoxadiazole (DAABD-Cl) and 7-chloro-2,1,3-benzoxadiazole-4-sulfonylaminoethyltrimethylammonium chloride (TAABD-Cl), are proposed for use in proteomics studies. Following derivatization of protein mixtures with these reagents, a series of standard processes of isolation, digestion, and identification of the proteins were performed utilizing high-performance liquid chromatography-fluorescence detection and tandem mass spectrometry with the probability-based protein identification algorithm. Both DAABD and TAABD derivatives were detected fluorometrically at the femtomole level and showed more than 100-fold improvement in sensitivity compared to the underivatized original compounds with an electrospray ionization ion trap mass spectrometer analysis. The modification of the MASCOT database search system memorized with the fragment information of a DAABD-attached Cys residue allowed the identification of the proteolytic peptide fragments of the derivatized bovine serum albumin (BSA) with an estimated 38% sequence coverage of BSA. Utilizing DAABD-Cl as a derivatization reagent, identification of several proteins was also possible in a soluble extract of *Caenorhabditis elegans* (10 μg of protein). Consequently, for identification of proteins in the complex matrixes of proteins, DAABD-Cl could be a more appropriate reagent than ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate as reported previously.

Although the conduct of a quantitative biological analysis is accepted as a routine in gathering genomic information, the approaches used in the analysis of DNA or mRNA do not provide exact information on the expressed proteins in real samples.[1,2] The establishment of a method for the identification of the expressed proteins is therefore essential.

In identifying proteins from protein matrixes, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used widely. This is then followed by proteolytic digestion. The sequences of proteins are usually determined through tandem mass spectrometry (MS/MS).[3−6] The separation by 2D-PAGE, however, is not only inappropriate for use in separating extremely acidic, basic, or hydrophobic proteins,[6−8] it needs a skillful technique to obtain reproducible results.[9] Therefore, for the quantitative analysis of the expressed proteins, different fluorescent cyanine dyes have been developed.[10,11] Because of the chemical properties of the dyes, protein labeling ratio with cyanine dyes was deliberately kept under 5%. Consequently, multiple labeling products were produced for each protein and afforded several spots on the gel.[9]

Multidimensional liquid chromatography (LC) has been used to separate the enzymatically digested peptides followed by identification by MS/MS.[12−15] Gygi et al. proposed a method using

* To whom correspondence should be addressed. Tel: +81-3-5400-2640. Fax: +81-3-5400-2640. E-mail: imai-kz@kyoritsu-ph.ac.jp.

[†] The University of Tokyo.

[‡] Musashino University.

(1) Chen, G.; Gharib, T. G.; Huang, C. C.; Taylor, J. M.; Misek, D. E.; Kardia, S. L.; Giordano, T. J.; Iannettoni, M. D.; Orringer, M. B.; Hanash, S. M.; Beer, D. G. *Mol. Cell. Proteomics* **2002**, *1*, 304−313.

(2) Griffin, T. J.; Gygi, S. P.; Ideker, T.; Rist, B.; Eng, J.; Hood, L.; Aebersold, R. *Mol. Cell. Proteomics* **2002**, *1*, 323−333.

(3) Page, M. J.; Amess, B.; Townsend, R. R.; Parekh, R.; Herath, A.; Brusten, L.; Zvelebil, M. J.; Stein, R. C.; Waterfield, M. D.; Davies, S. C.; O'Hare, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12589−12594.

(4) Srisomsap, C.; Subhasitanont, P.; Otto, A.; Mueller, E. C.; Punyarit, P.; Wittmann-Liebold, B.; Svasti, J. *Proteomics* **2002**, *2*, 706−712.

(5) Jungblut, P. R.; Zimny-Arndt, U.; Zeindl-Eberhart, E.; Stulik, J.; Koupilova, K.; Pleissner, K. P.; Otto, A. Muller, E. C.; Sokolowska-Kohler, W.; Grabher, G.; Stoffler, G. *Electrophoresis* **1999**, *20*, 2100−2110.

(6) Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9390−9395.

(7) Molly, M. *Anal. Biochem.* **2000**, *280*, 1−10.

(8) Santoni, V.; Molly, M.; Rabiloud, T. *Electrophoresis* **2000**, *21*, 1054−1070.

(9) Corthals, G. L.; Wasinger, V. C.; Hochstrasser, D. F.; Sanchez, J. C. *Electrophoresis* **2000**, *21*, 1104−1115.

(10) Ünlü, M.; Morgan, M. E.; Minden, J. S. *Electrophoresis* **1997**, *18*, 2071−2077.

(11) Tonge, R.; Shaw, J.; Middleton, B.; Rowlinson, R.; Rayner, S.; Young, J.; Pognan, F.; Hawkins, E.; Currie, I.; Davison, M. *Proteomics* **2001**, *1*, 377−396.

(12) Wagner, K.; Racaityte, K.; Unger, K. K.; Miliotis, T.; Edholm, L. E.; Bischoff, R.; Marko-Varga, G. *J. Chromatogr., A* **2000**, *893*, 293−305.

(13) Davis, M. T.; Beierle, J.; Bures, E. T.; McGinley, M. D.; Mort, J.; Robinson, J. H.; Spahr, C. S.; Yu, W.; Luethy, R.; Patterson, S. D. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *752*, 281−291.

(14) Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. *Nat. Biotechnol.* **1999**, *17*, 676−682.

10.1021/ac034840i CCC: $27.50
Published on Web 01/07/2004

isotope-coded affinity tags (ICAT) reagents, biotin and a mass-differentiated group to quantify the proteins from the protein mixtures. The process consists of labeling the proteins with the reagents, proteolytic digestion, isolation of the tagged peptides (by use of an avidin column), liquid chromatogreaphic separation, and finally identification and quantification by MS/MS.[16−19] As an alternative approach, tandem mass tags have been developed.[20] The tandem mass tags are composed of a sensitization group of guanidino functionality and a mass normalization group to derivatize proteins. The relative abundance of proteins is measured using MS/MS-based detection. However, the molecular masses of ICAT reagents and tandem mass tags are relatively high, which might cause the less soluble derivatized proteins to be absorbed onto the tubes and columns. It was also true for the cleavable reagents now in use, (*S*)-pentafluorophenyl[tris(2,4,6-trimethoxyphenyl)phosphonium] acetate bromide, bearing high molecular mass.[21−23] Therefore, we developed water-soluble, small-mass derivatization reagents for proteomics studies.

In a previous paper,[24] we reported a method for proteomics studies. The protein mixtures were first derivatized with a water-soluble fluorogenic reagent, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F),[25] followed by isolation, digestion of the modified proteins, and identification of the proteins. The identification of these proteins was performed by utilizing high-performance liquid chromatography (HPLC)-fluorescence detection and electrospray ionization (ESI)-MS/MS with the probability-based protein identification algorithm. The derivatized proteins were highly fluorescent, with long excitation (380 nm) and emission wavelengths (520 nm), and suitable for femtomole detection owing to the 2,1,3-benzoxadiazole (benzofurazan) skeleton.[26,27] In addition, the derivatives with SBD-F were hydrophilic and suitable for their isolation by HPLC. Using the proposed method, 11 altered proteins were identified in the islets of Langerhans in rats pretreated with dexamethazone. By using this method, however, the SBD-peptides after digestion with enzymes could not be detected by MS to our satisfaction, due probably to the negatively charged sulfonyl group in the SBD moiety.

For matrix-assisted laser desorption/ionization (MALDI)-MS[21−23,28−32] or ESI-MS,[32−37] the proteins with positive charges were proved to be highly detectable because of their easy ionization and simple fragmentation.[38] Thus, the peptides were modified to have a positively charged group attached to the N-terminus to simplify MS/MS spectra.[29−31]

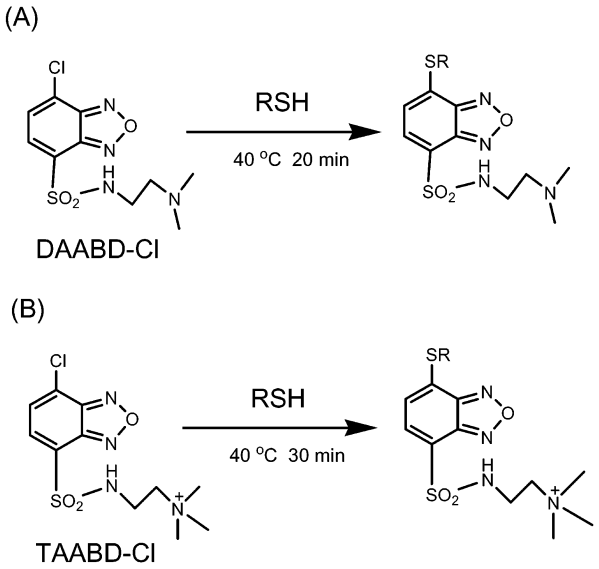

Figure 1. Chemical structures of DAABD-Cl and TAABD-Cl and the derivatization reaction for thiols.

In this paper, to improve the detectability of the modified proteins in MS, we develop the two water-soluble and positively charged fluorogenic derivatization reagents for thiols instead of SBD-F, i.e., 4-(dimethylaminoethylaminosulfonyl)-7-chloro-2,1,3-benzoxadiazole (DAABD-Cl) and 7-chloro-2,1,3-benzoxadiazole-4-sulfonylaminoethyltrimethylammonium chloride (TAABD-Cl). The chemical structures of DAABD-Cl and TAABD-Cl and their derivatives are shown in Figure 1. The DAABD and TAABD derivatives are positively charged in solution and proved to be sensitively detected fluorometrically and mass spectrometrically. The applicability of DAABD-Cl is also described for identification of an authentic bovine serum albumin (BSA) and proteins in a soluble extract of *Caenorhabditis elegans*.

EXPERIMENTAL SECTION

Material and Reagents. 4-Chlorosulfonyl-7-chloro-2,1,3-benzoxadiazole and *N*,*N*-dimethylethylenediamine were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Aminoethyltrimethylam-

(15) Wolters, D. A.; Washburn, M. P.; Yates, J. R., 3rd. *Anal. Chem.* **2001**, *73*, 5683−5690.
(16) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994−999.
(17) Han, D. K.; Eng, J.; Zhou, H.; Aebersold, R. *Nat. Biotechnol.* **2001**, *19*, 946−951.
(18) Zhang, R.; Sioma, C. S.; Wang, S.; Regnier, F. E. *Anal. Chem.* **2001**, *73*, 5142−5149.
(19) Griffin, T. J.; Han, D. K.; Gygi, S. P.; Rist, B.; Lee, H.; Aebersold, R.; Parker, K. C. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 1238−1246.
(20) Schlosser, A.; Lehmann, W. D. *J. Mass Spectrom.* **2000**, *35*, 1382−1390.
(21) Shen, T. L.; Huang, Z. H.; Laivenieks, M.; Zeikus, J. G.; Gage, D. A.; Allison, J. *J. Mass Spectrom.* **1999**, *34*, 1154−1165.
(22) Huang, Z. H.; Wu, J.; Roth, K. D. W.; Yang, Y.; Gage, D. A.; Watson, J. T. *Anal. Chem.* **1997**, *69*, 137−144.
(23) Adamczyk, M.; Gebler, J.; Wu, J. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1413−1422.
(24) Toriumi, C.; Imai, K. *Anal. Chem.* **2003**, *75*, 3725−3730.
(25) Imai, K.; Toyo'oka, T.; Watanabe, Y. *Anal. Biochem.* **1983**, *128*, 471−473.
(26) Imai, K.; Uzu, S.; Toyo'oka, T. *J. Pharm. Biomed. Anal.* **1989**, *7*, 1395−1403.
(27) Uchiyama, S.; Santa, T.; Okiyama, N.; Toriba, A.; Imai, K. *Biomed. Chromatogr.* **2001**, *15*, 295−318.
(28) Broberg, S.; Broberg, A.; Duus, J. O. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1801−1805.
(29) Peters, E. C.; Horn, D. M.; Tully, D. C.; Brock, A. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2387−2392.
(30) Spengler, B.; Luetzenkirchen, F.; Metzger, S.; Chaurand, P.; Kaufmann, R.; Jeffery, W.; Bartlet-Jones, M.; Pappin, D. J. C. *Int. J. Mass Spectrom.* **1997**, *169*, 127−140.
(31) Huang, Z. H.; Shen, T. L.; Wu, J. A.; Gage, D. A.; Watson, J. T. *Anal. Biochem.* **1999**, *268*, 305−317.
(32) Stults, J. T.; Lai, J.; McCune, S.; Wetzel, R. *Anal. Chem.* **1993**, *65*, 1703−1708.
(33) Cech, N. B.; Enke, C. *Mass Spectrom. Rev.* **2001**, *20*, 362−387.
(34) Yoshino, K.; Takao, T.; Murata, H.; Shimonishi, Y. *Anal. Chem.* **1995**, *67*, 4028−4031.
(35) Okamoto, M.; Takahashi, K. I.; Doi, T. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 641−643.
(36) Suzuki, S.; Kakehi, K.; Honda, S. *Anal. Chem.* **1996**, *68*, 2073−2083.
(37) Quirke, J. M.; Van Berkel, G. J. *J. Mass Spectrom.* **2001**, *36*, 1294−1300.
(38) Roth, K. D.; Huang, Z. H.; Sadagopan, N.; Watson, J. T. *Mass Spectrom. Rev.* **1998**, *17*, 255−274.

monium chloride was from Aldrich Co. (Milwaukee, WI). Acetonitrile and methanol, both of HPLC grade, dichloromethane, and cysteine were purchased from Kanto Chemical Co. (Tokyo, Japan). Homocysteine, tris(2-carboxethyl)phosphine hydrochloride (TCEP), and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Formic acid, trifluoroacetic acid, ammonium bicarbonate, and glutathione (reduced form) was from Wako Pure Chemicals (Osaka, Japan). Silica gel was purchased from Merck (Darmstadt, Germany), and guanidine hydrochloride was from Nacalai Tasque (Kyoto, Japan). Trypsin was obtained from Promega (Madison, WI). 3-[(3-Cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPS) and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) were from Dojindo Laboratories (Kumamoto, Japan). Vasopressin, oxytocin, calcitonin, and amylin were purchased from the Peptide Institute (Osaka, Japan). All other reagents used were of guaranteed reagent grade and used without further purification.

Apparatus. Proton nuclear magnetic resonance (^{1}H NMR) spectra were acquired using a JEOL LA-500 spectrometer (Tokyo, Japan) with tetramethylsilane as an internal standard in CD_3OD. Mass spctra were obtained using an ESI ion trap mass spectrometer (Esquire 3000+, Bruker Daltonics, Billerica, MA). Melting points were measured on a Yanagimoto Micro Point Apparatus (Tokyo, Japan). Fluorescence spectra were measured using a Hitachi-4500 fluorescent spectrometer (Tokyo, Japan).

Synthesis of 4-(Dimethylaminoethylaminosulfonyl)-7-chloro-2,1,3-benzoxadiazole. 4-Chlorosulfonyl-7-chloro-2,1,3-benzoxadiazole (126.53 mg) was dissolved in CH_3CN (6 mL). After the addition of *N*,*N*-dimethylethylenediamine (88.8 mg) and triethylamine (36 μL), the mixture was stirred at room temperature for 10 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel using $CH_2Cl_2-CH_3OH$ (9:1) to afford DAABD-Cl (120.2 mg, 87.4%) as a yellow powder: mp, 107 °C; ^{1}H NMR δ_H 7.94 (1H, d, $J = 7.5$ Hz), 7.65 (1H, d, $J = 7.5$ Hz), 3.06 (2H, t, $J = 6.7$ Hz), 2.30 (2H, t, $J = 6.7$ Hz), 2.02 (6H, s); ESI-MS m/z 305 $(M + H)^+$. Anal. Calcd for $C_{10}H_{13}O_3N_4SCl$: C, 39.41; H, 4.30; N, 18.38. Found: C, 39.24; H, 4.40; N, 18.14.

Synthesis of 7-Chloro-2,1,3-benzoxadiazole-4-sulfonylaminoethyl Trimethylammonium Chloride. 4-Chlorosulfonyl-7-chloro-2,1,3-benzoxadiazole (126.53 mg) was dissolved in CH_3CN (6 mL). After the addition of aminoethyltrimethylammonium chloride (87.55 mg) dissolved in H_2O (2 mL) and triethylamine (73 μL), the mixture was stirred at room temperature for 20 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on a column of TSKgel ODS-80 TMG (20 × 75 mm i.d.) using the HPLC system. The HPLC system consisted of a pump (L-7100, Hitachi, Tokyo, Japan), a column, and an ultraviolet detector (UV-970, Jasco, Tokyo, Japan). The UV detection was carried out at a wavelength of 254 nm. Mobile phases: the eluent (A) 0.1% trifluoroacetic acid and the eluent (B) water/CH_3CN/trifluoroacetic acid (30/70/0.1). The gradient elution was carried out from 10 to 100% (B) over 25 min with a flow rate of 3.0 mL/min. The fraction of TAABD-Cl including 7-chloro-2,1,3-benzoxadiazole-4-sulfonate (SBD-Cl)[39] was eluted at 19.8 min, which was collected, concentrated, and purified to remove SBD-Cl on a column of TSKgel DEAE-5PW (75 × 2.0 mm i.d.) using the HPLC system. Mobile phases: the eluent (A) 0.1% trifluoroacetic acid and the eluent (B) water/CH_3CN/trifluoroacetic acid (30/70/0.1). The gradient elution was performed from 40 to 60% B over 10 min at a flow rate of 0.5 mL/min. The fraction containing TAABD-Cl was eluted at 4.5 min and evaporated to afford the trifluoroacetic acid adduct of TAABD-Cl (127.2 mg, 58.8%) as a white powder: mp, 108−109 °C; ^{1}H NMR δ_H 8.01 (1H, d, $J = 7.3$ Hz), 7.69 (1H, d, $J = 7.3$ Hz), 3.46−3.48 (4H, m), 3.12 (9H, s); ESI-MS m/z 319 $(M)^+$. Anal. Calcd for $C_{13}H_{16}O_5N_4SClF_3$: C, 36.08; H, 3.72; N, 12.94. Found: C, 35.40; H, 3.52; N, 12.52.

Time Course for the Derivatization Reaction of Thiols with DAABD-Cl and TAABD-Cl. A 50-μL aliquot each of the fluorogenic reagent solution (1 mg/mL), DAABD-Cl or TAABD-Cl in 100 mM borate buffer containing 5 mM Na_2EDTA (pH 9) was mixed with the same volume of a 10 μM mixture of cysteine, homocysteine, and glutathione. The reaction mixture was incubated at 40 °C for 10−120 min, and the reaction was stopped with 100 μL of 0.1% formic acid. After the reaction, a 5-μL aliquot of the reaction mixture was injected into the HPLC system. The HPLC system consisted of a pump (L-7100, Hitachi), a separation column TSKgel 120-T QA (250 × 4.6 mm i.d.) (Tosoh, Tokyo, Japan), and a fluorescence detector (FL-2025, Jasco). The fluorescence detection was carried out at 387 and 508 nm for the excitation and emission wavelengths, respectively. The mobile phase was 150 mM phosphate buffer/CH_3CN (94/6), and the flow rate was 1.0 mL/min.

Measurement of Fluorescence Spectra. The solutions of the fluorescent derivatives of cysteine, homocysteine, and glutathione (10 μM) in 150 mM phosphate buffer/CH_3CN (94/6) were used to obtain the fluorescence spectra and the maximum excitation and emission wavelengths.

Fluorescence Characteristics of DAABD Derivatives. Fluorescence intensities of the derivative of glutathione (20 μM) with DAABD-Cl in various pH solutions (Britton−Robinson buffer) were measured at maximum excitation and emission wavelengths. Solution A (100 mL) for the Britton−Robinson buffer consisted of 232 μL of 85% phosphoric acid, 240 μL of acetic acid, and 247 mg of boric acid. Solution B (100 mL) was 0.8 g of sodium hydroxide in water.

Identification and Detection of DAABD and TAABD Derivatives of Thiols Using LC−MS. The derivatization reaction was performed with DAABD-Cl or TAABD-Cl according to the optimized condition (see Results and Discussion). The reaction mixture of cysteine, homocysteine, and glutathione was directly injected into the HPLC-MS system. Chromatography was performed using a HP 1090 series II system (Hewlett-Packard GmbH) and a separation column, Cadenza TC-18 column (12-nm pores of silica, 100 × 2.0 mm i.d.) (Imtakt, Kyoto, Japan). Mobile phase: the eluent (A) 0.1% formic acid and the eluent (B) water/CH_3CN/formic acid (50/50/0.1). The gradient elution was carried out from 0 to 100% B over 15 min with a flow rate of 0.2 mL/min.

Derivatization Reaction of Peptides and BSA with DAABD-Cl and TAABD-Cl. A 20-μL aliquot of 250 nM peptide solution (vasopressin, oxytocin, calcitonin, and amylin) or BSA (final concentration 50 nM) was mixed with the same volume of the respective 0.5−5.0 mM TCEP, 17.5 mM derivatization reagent (DAABD-Cl or TAABD-Cl), 10 mM Na_2EDTA, and 50 mM

(39) Andrews, J. L.; Ghosh, P.; Ternai, B.; Whitehouse, M. W. *Arch. Biochem. Biophys.* **1982**, *214*, 386−396.

CHAPS. Each reagent was dissolved in 100 mM borate buffer (pH 9.0) containing 6.0 M guanidine. Each reaction mixture was incubated at 40 °C for 30−120 min, and the reaction was stopped with 200 μL of 0.1% formic acid. After the reaction, a 30-μL aliquot of the reaction mixture was injected into the HPLC system. The HPLC system consisted of a pump (L-7100, Hitachi), a column of Capcellpak C8 SG 300 (30-nm pore size, 100 × 2.0 mm i.d., Shiseido, Tokyo, Japan), and a fluorescence detector (FL-2025, Jasco). Fluorescence detection was carried out at 387 and 508 nm for the excitation and emission wavelengths, respectively. Mobile phases: the eluent (A) 0.1% trifluoroacetic acid and the eluent (B) water/CH_3CN/trifluoroacetic acid (30/70/0.1). The gradient elution was carried out from 0 to 100% B over 30 min with a flow rate of 0.5 mL/min.

Identification of BSA Derivatized with DAABD-Cl Using HPLC and Tandem Mass Spectrometry. The eluate of the BSA derivative was concentrated to 60 μL under reduced pressure. Twenty microliters of the residue (∼2.0 pmol) was diluted with 180 μL of 50 mM ammonium bicarbonate solution (pH 7.8) containing 2 μg/mL trypsin and 1.0 mM calcium chloride, and the resultant mixture was incubated for 2 h at 37 °C. The ploteolytic peptide mixture was directly subjected to LC−MS/MS using an ESI ion trap mass spectrometer.

Chromatography was performed using a HP 1090 series II system and a column of Cadenza TC-18 column (12-nm pores silica, 100 × 2.0 mm i.d.). Mobile phases: eluent (A) 1.0 mM ammonium formate and eluent (B) 1.0 mM ammonium formate/CH_3CN (50/50). The gradient elution was carried out from 0 to 100% B over 60 min with a flow rate of 0.2 mL/min. The identification of the protein was performed against the NCBInr database with the MASCOT (Matrix Science Ltd.) database-searching algorithm memorizing the DAABD-attached thiol residue of cysteine.

Derivatization and Identification of *C. elegans* Proteins with DAABD-Cl. *C. elegans* (strain Bristol N2) was grown on NGM agar, using the OP50 strain of *Escherichia coli* as a food source at 20 °C and separated from bacteria by flotation on M9 buffer. After washing with M9 buffer twice, the worms were stored at −80 °C until use. Then, they were suspended with an equal volume of 10 mM CHAPS and lysed by sonication on ice. The soluble fraction was collected by centrifugation at 10 000 rpm for 5 min at 4 °C. The supernatant was stocked as a soluble fraction at −20 °C. Protein concentration of the fraction was determined by the Bradford method[40] using BSA as a standard. About 20 μL (100 μg of protein) of the supernatant was mixed with the same volume of the respective 2.5 mM TCEP, 17.5 mM DAABD-Cl, 10 mM Na_2EDTA, and 50 mM CHAPS in 100 mM borate buffer (pH 9.0) containing 6.0 M guanidine. After the reaction mixture was incubated at 40 °C for 30 min, the reaction was stopped with 200 μL of 0.1% formic acid, and then a 30-μL aliquot of the reaction mixture (10 μg of protein) was injected into the HPLC system. HPLC was performed with the same condition as described above except using a column of RP for protein (30-nm pore size, 250 × 4.6 mm i.d.) (Imtakt). Mobile phases: the eluent (A) 0.1% trifluoroacetic acid and the eluent (B) water/CH_3CN/trifluoroacetic acid (70/30/0.1). The gradient system was from 70 to 30% B over 100 min with a flow rate of 0.25 mL/min.

(40) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.

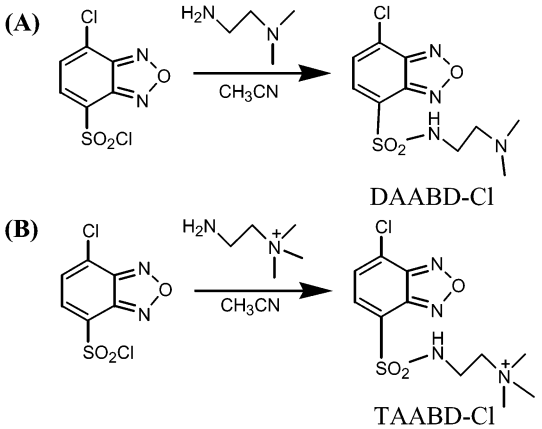

Figure 2. Synthetic routes for DAABD-Cl and TAABD-Cl.

For the identification, several peak fractions of the fluorescent protein derivatives were isolated and concentrated to 10 μL under reduced pressure. Each fraction was diluted with 90 μL of 50 mM ammonium bicarbonate solution (pH 7.8) containing 2 μg/mL trypsin and 1.0 mM calcium chloride, and the resultant mixture was incubated for 2 h at 37 °C. Each ploteolytic peptide mixture was directly subjected to LC-MS/MS using an ESI ion trap mass spectrometer. The chromatographic conditions are the same as described above in Identification of BSA Derivatized with DAABD-Cl Using HPLC and Tandem Mass Spectrometry.

RESULTS AND DISCUSSION

Design and Synthesis of DAABD-Cl and TAABD-Cl. As described in the introduction, the benzofurazan reagents used in the present work have a moiety of positive charge instead of negative charge as in SBD-F. Therefore, the sulfonic acid moiety in SBD-F was replaced with a dimethyl- or trimethylaminoethylaminosulfonyl moiety. Two corresponding new reagents, DAABD-Cl and TAABD-Cl, were synthesized. As shown in Figure 2, the structures of these reagents are similar to 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F)[41] and 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F).[42] Since, in our preliminary experiment, ABD-F and DBD-F were more reactive than SBD-F, both ABD-F and DBD-F decomposed in the presence of reducing agent (TCEP),[43] whereas SBD-F did not. Therefore, we planned to make the reagents with the less reactive chlorine atom instead of fluorine atom at the 7 position of the benzofrazan structure in DAABD and TAABD structures, and thus, DAABD-Cl and TAABD-Cl were designed. As expected, they were nonfluorescent in aqueous organic solvent such as water/CH_3CN or water/CH_3OH.

Reaction Conditions and Reactivity of the Derivatization Reaction of DAABD-Cl and TAABD-Cl for Thiols. Time course studies on the derivatization reaction of small molecular mass thiols (cysteine, homocysteine, glutathione) with DAABD-Cl or TAABD-Cl were performed at 40 °C (pH 9.0). As shown in Figure 3, the fluorescence emerged and its intensity reached the maximum at 20 and 30 min for DAABD-Cl and TAABD-Cl,

(41) Toyo'oka, T.; Imai, K. *Anal. Chem.* **1985**, *57*, 1931−1937.
(42) Toyo'oka, T.; Suzuki, T.; Saito, Y.; Uzu, S.; Imai, K. *Analyst* **1989**, *114*, 413−419.
(43) Lauren, S. L.; Treuheit, M. J. *J. Chromatogr., A* **1998**, *798*, 47−54.

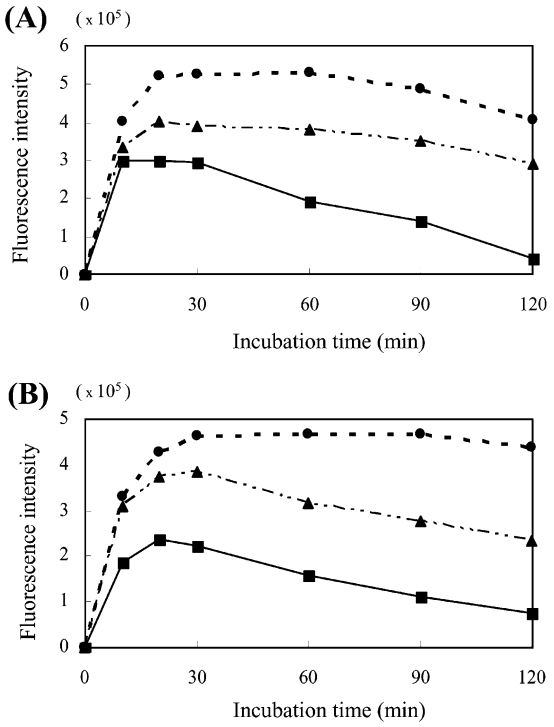

Figure 3. Time course for the derivatization reactions of thiols with (A) DAABD-Cl and (B) TAABD-Cl. Symbols: ■, cysteine; ●, homocysteine; ▲, glutathione.

respectively, indicating that the reagents rapidly reacted with thiols under these mild conditions to afford fluorescence. Since SBD-F requires 120 min for the derivatization reaction at 40 °C (data not shown), it can be concluded that DAABD-Cl and TAABD-Cl are more reactive than SBD-F and that DAABD-Cl was more reactive than TAABD-Cl.

As mentioned above, as the structures of DAABD-Cl and TAABD-Cl are similar to ABD-F and DBD-F, it was expected that they would decompose with TCEP. However, there were no decomposition of DAABD-Cl and TAABD-Cl in the presence of TCEP (data not shown). Furthermore, the derivatives with these reagents were also stable with TCEP, whereas ABD-cysteine containing peptide was delabeled in the presence of reducing reagent (dithiothreitol).[43]

Fluorescence Characteristics of Thiol Derivatives with DAABD-Cl and TAABD-Cl. The fluorescence spectra of the thiol derivatives showed that the maximum excitation and emission wavelengths were respectively 387 and 508 nm for DAABD derivatives but 386 and 507 nm for TAABD derivatives.

The relationship between the medium pH and the fluorescence intensities of the DAABD derivative of glutathione is shown in Figure 4. In the acidic and neutral conditions (at under pH 7), the intensity was more than 14 times higher than those in the basic conditions (at pH 11 and above). Thus, the fluorescence intensity of the derivative decreases as the solution becomes more basic. We therefore concluded that, for a more sensitive detection of the derivatives, the detection should be performed at a pH under 7. It is plausible that the nitrogen atom at the dimethylamino group of DAABD-Cl may be deprotonated in the basic condition and quenching of the fluorescence occurred by an electron transfer from nitrogen atom in the excited state of the fluorophore. The observation is in agreement with the so-called photoinduced

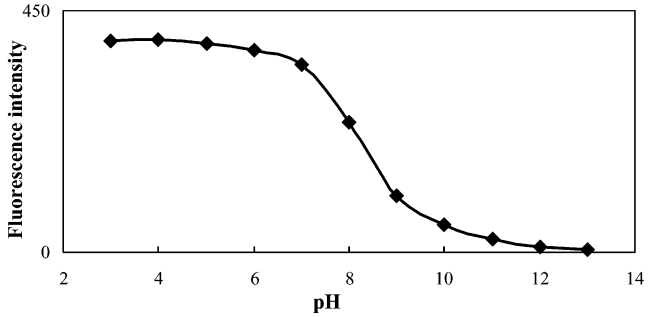

Figure 4. pH profile of the fluorescence intensity of the derivative of glutathione with DAABD-Cl.

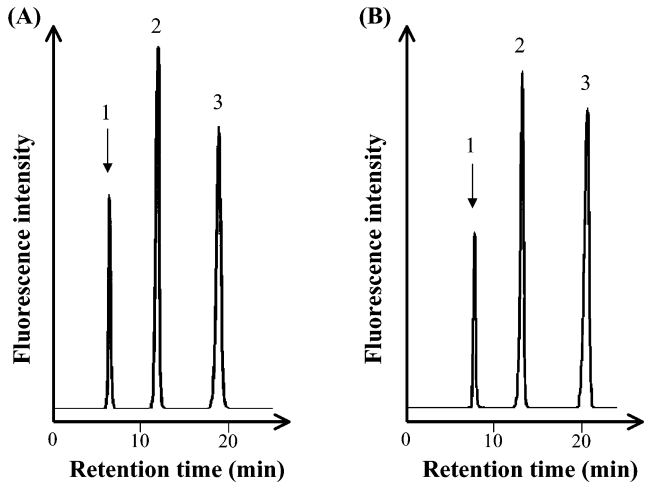

Figure 5. Chromatograms obtained from the thiols derivatized with (A) DAABD-Cl and (B) TAABD-Cl (5 pmol). Peaks: 1, cysteine; 2, homocysteine; 3, glutathione. Chromatographic conditions are described in the Experimental Section.

electron transfer.[44,45] Accordingly, in acidic and neutral conditions, we presumed the nitrogen atom was protonated.

Linearity and Detection Limits for DAABD and TAABD Derivatives. The chromatograms (Figure 5) obtained from the derivatives of cysteine, homocysteine, and glutathione with DAABD-Cl or TAABD-Cl showed a single peak for each of the derivatives, and no interfering peaks were observed. Neither DAABD-Cl nor TAABD-Cl elution peaks appeared because the reagents themselves do not fluoresce. The detection limits (signal-to-noise ratio 3) for cysteine, homocysteine, and glutathione were 110, 62.8, and 81.2 fmol, respectively, for the DAABD derivatives and 147, 70.4, and 85.2 fmol, respectively, for the TAABD derivatives. The calibration curves for each derivative were linear over the range from 100 fmol to 100 pmol, and the correlation coefficients were greater than 0.999.

Identification and Detection of DAABD and TAABD Derivatives of Thiols Using LC−MS. The derivatization reaction mixtures of thiols with DAABD-Cl or TAABD-Cl were subjected to HPLC with an ESI ion trap mass spectrometer. The mass spectra for each derivative of DAABD-Cl or TAABD-Cl (Figure 6) showed that the derivatives of DAABD-Cl were detected as the base ion peaks of $m/z = 390$ $(M + H)^+$, 404 $(M + H)^+$, and 576 $(M + H)^+$ for cysteine (MW = 121), homocysteine (MW =

(44) Uchiyama, S.; Santa, T.; Imai, K. *Analyst* **2000**, *125*, 1839−1845
(45) Onoda, M.; Uchiyama, S.; Santa, T.; Imai, K. *Anal. Chem.* **2002**, *74*, 4089−4096

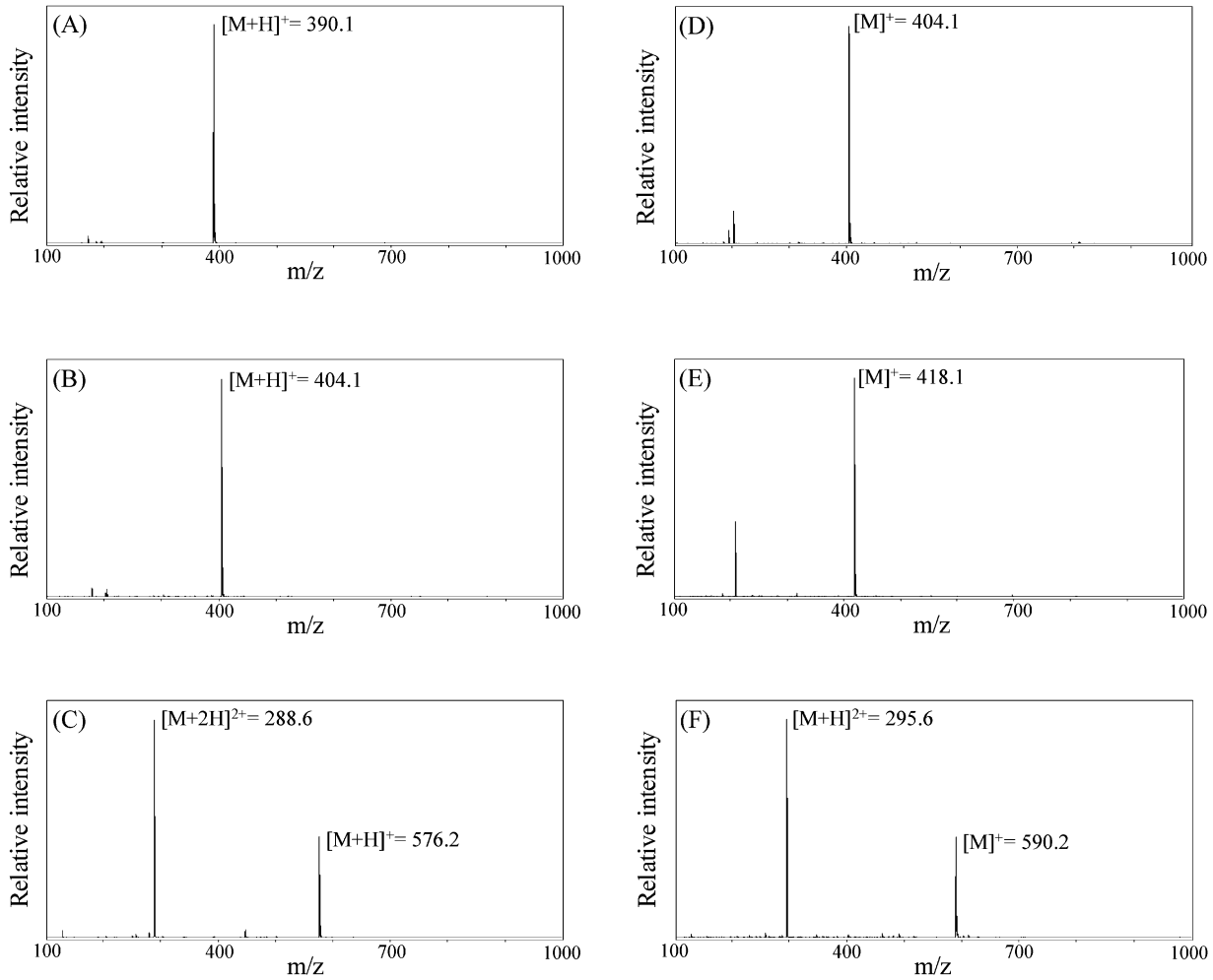

Figure 6. Positive-ion ESI mass spectra of the derivatized thiols (5 pmol). (A) cysteine with DAABD-Cl, (B) homocysteine with DAABD-Cl, (C) glutathione with DAABD-Cl, (D) cysteine with TAABD-Cl, (E) homocysteine with TAABD-Cl, and (F) glutathione with TAABD-Cl.

135), and glutathione (MW = 307), respectively. In contrast, the derivatives of TAABD-Cl were detected as the base ion peaks of m/z = 404 $(M)^+$, 418 $(M)^+$, and 590 $(M)^+$ for cysteine, homocysteine, and glutathione, respectively.

The ratios of intensities of base peak ion of each derivative with DAABD-Cl to the corresponding underivatized cysteine, homocysteine, and glutathione were 3.0×10^3, 2.3×10^2, and 2.1×10^2, respectively, whereas in case of TAABD-Cl, the ratios were 2.0×10^3, 1.6×10^2, and 1.7×10^2 for cysteine, homocysteine, and glutathione, respectively. In contrast, the respective ratios of the derivatives with SBD-F were 23, 4.0, and 1.6 for cysteine, homocysteine, and glutathione, suggesting that the derivatization with DAABD-Cl or TAABD-Cl resulted in a significant enhancement (up to 100−3000-fold) of detectability as compared with that with SBD-F. Therefore, DAABD-Cl and TAABD-Cl are more sensitive reagents in MS detection than SBD-F.

Derivatization Reaction of Peptides and BSA with DAABD-Cl and TAABD-Cl. The derivatization conditions with the reagents were investigated with BSA as a model protein. BSA was dissolved in 6.0 M guanidine hydrochloride and 2.0 mM EDTA (to avoid the oxidation of thiols). With DAABD-Cl (3.5 mM), the addition of TCEP (0.1−1.0 mM) induced a dose-dependent reaction yield, with the maximum obtained at 0.5 mM TCEP, whereas, with TAABD-Cl, TCEP induced only some interfering peaks at all concentrations. Therefore, DAABD-Cl was selected hereafter to derivatize peptides and proteins.

In the preliminary experiments, reaction temperatures higher than 50 °C resulted in a decrease of peak intensity. Thus, 40 °C was selected for the reaction. Likewise, in an examination of the concentrations of DAABD-Cl, 3.5 mM was selected to achieve complete derivatization. The reaction mixture was incubated at 40 °C for 10−120 min, and the fluorescence intensity reached the maximum at 30 min (data not shown).

Considering the data obtained in preliminary experiments, the following derivatization conditions were selected: 100 mM borate buffer (pH 9.0) containing 6.0 M guanidine hydrochloride with 3.5 mM DAABD-Cl, 1.0 mM EDTA and 10 mM CHAPS at 40 °C for 30 min. Under these conditions, the mixtures of peptides and a protein, i.e., vasopressin, oxytocin, calcitonin, amylin, and BSA, were derivatized without precipitation, and the chromatograms showed a single fluorescent peak for each compound tested (Figure 7). Good linearities were obtained between the peak areas and the amounts of compounds from 10 to 1000 fmol ($r > 0.999$). The detection limits for vasopressin, oxytocin, calcitonin, amylin, and BSA were 7.0, 4.5, 5.0, 4.0, and 0.5 fmol, respectively.

Identification of BSA Derivatized with DAABD-Cl Using HPLC and Tandem Mass Spectrometry. The derivatized BSA with DAABD-Cl was digested with trypsin. A part of the resulting

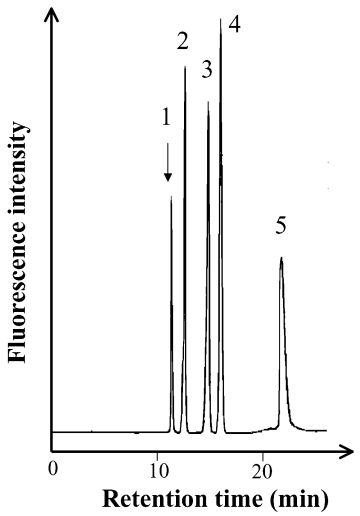

Figure 7. Representative chromatogram of several peptides and BSA derivatized with DAABD-Cl. Peaks: 1, vasopressin (500 fmol); 2, oxytocin (500 fmol); 3, calcitonin (500 fmol); 4, amylin (500 fmol); 5, BSA (50 fmol). Chromatographic conditions are described in the Experimental Section.

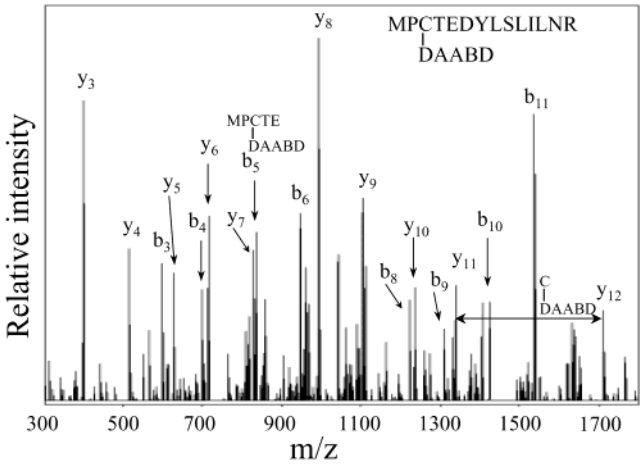

Figure 8. Positive-ion ESI MS/MS spectrum of the peptide fragment obtained from the digested BSA derivatized with DAABD-Cl (~0.5 pmol, m/z = 969.0).

mixture of peptides (~0.5 pmol each) was separated by HPLC and detected using a fluorescence detector or ESI ion trap mass spectrometer to perform the MS/MS analysis. The 25 fluorescent peptides were detected on the HPLC chromatogram in agreement with the 25 peaks of theoretical expectation, considering that the derivatization with DAABD-Cl of the protein did not disturb the conventional tryptic digestion. Figure 8 shows an example of ESI-MS/MS spectra, on which b- and y-type ions were predominant, indicating that there were no significant unexpected MS fragmentation. Utilizing the modified MASCOT database search system, memorized with the fragment information of the cysteine residue attached to DAABD (from 103 to 390), the sequence information of 21 proteolytic peptides [9 cysteine-containing peptides (m/z = 544.3, 589.2, 629.0, 634.3, 698.5, 828.4, 902.2, 969.0, 1060.5) and 12 non-cysteine-containing peptides] was obtained from the derivatized BSA, which represents 232 amino acids residues, an estimated 38% coverage of BSA. Therefore, we concluded DAABD derivatives were applicable to HPLC−MS/MS analysis of proteins.

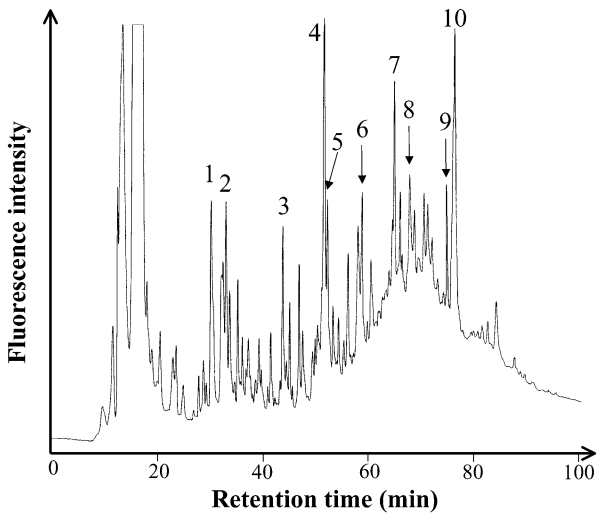

Figure 9. Chromatogram of the proteins (10 μg) in the soluble fraction of *C. elegans* derivatized with DAABD-Cl. Peaks: 1, ribosomal protein S3a (MW = 28 942); 2, calreticulin precursor (MW = 45 588); 3, ribosomal protein L1 (MW = 38 635); 4, elongation factor 1-α (MW = 50 636); 5, malate dehydrogenase (MW = 35 098); 6, 40S ribosomal protein (MW = 22 044); 7, vitellogenin (MW = 193 098); 8, arginine kinase (MW = 41 969); 9, HSP-1 heat shock 70-kDa protein A (MW = 69 680), and 10, ribosomal protein L7Ae (MW = 13 992). Chromatographic conditions are described in the Experimental Section.

Derivatization and Identification of *C. elegans* Proteins with DAABD-Cl. It is known that *C. elegans* is good material for proteomics studies analysis because this is the first multicellular model animal for which the genome sequence was determined,[46] and for which the amino acid sequence database is available.[47] So far, Kaji et al. have reported that 69 spots were separated by 2D-PAGE and identified by in-gel digestion followed by MALDI-MS,[48] and Schrimpf et al. identified 152 proteins through 2D-PAGE.[49]

Figure 9 shows a chromatogram of proteins (~10 μg) obtained from a soluble fraction of *C. elegans* derivatized with DAABD-Cl. After isolation, tryptic digestion, and LC−MS/MS identification of the arbitrary selected peak fractions, we identified 10 proteins: (1) ribosomal protein S3a (MW = 28 942); (2) calreticulin precursor (MW = 45 588); (3) ribosomal protein L1 (MW = 38 635); (4) elongation factor 1-α (MW = 50 636); (5) malate dehydrogenase (MW = 35 098); (6) 40S ribosomal protein (MW = 22 044); (7) vitellogenin (MW = 193 098); (8) arginine kinase (MW = 41 969); (9) HSP-1 heat shock 70-kDa protein A (MW = 69 680), and (10) ribosomal protein L7Ae (MW = 13 992). Although, in the present experiment, only arbitrary selected 10 proteins were identified (Figure 9), the better separation on an elaborate column could enable identification of more proteins in a single run of the derivatization procedure.

In conclusion, compared to SBD-F, the reagents presented in this work afforded a more sensitive mass spectrometric detection

(46) T. C.e. S. Consortium *Science* **1998**, *282*, 2012−2018.
(47) http://www.sanger.ac.uk/Projects/C_elegans/wormpep.
(48) Kaji, H.; Tsuji, T.; Mawuenyega, K. G.; Wakamiya, A.; Taoka, M.; Isobe, T, *Electrophoresis* **2000**, *21*, 1755−1765.
(49) Schrimpf, S. P.; Langen, H.; Gomes, A. V.; Wahlestedt, T, *Electrophoresis* **2001**, *22*, 1224−1232.
(50) Toriumi, C.; Santa, T.; Imai, K. *Proc. Japan Acad., Ser. B.* **2003**, *79*, 137−140.

of the derivatives of the protein and thus are more appropriate reagents for proteomic studies. DAABD-Cl in particular would be useful for the identification of proteins in the complex matrixes of proteins. Furthermore, it could be ideal for quantitative proteomics studies when two different reagents bearing similar structures yet with different fluorescence characteristics, such as benzoselenadiazole and benzothiadiazole reagents, are used. For example, as the maximum emission (340 nm) and excitation wavelengths (542 nm) of 7-halogeno-2,1,3-benzoselenadiazole-4-sulfonate derivatives[50] are different from those of SBD derivatives, two different samples are derivatized with the respective reagents, mixed together, and detected fluometrically with each of the suitable wavelengths.

ACKNOWLEDGMENT

The authors thank Dr. Takeshi Iwatsubo and Mr. Tomoki Kuwahara, The University of Tokyo, for supplying the worm with kind technical guidance. We also thank Dr. Chang-Kee Lim, MRC Bioanalytical Science Group and Dr. Chiho Lee, Yamato Scientific Co. Ltd., for their kind suggestions and valuable discussion. We thank Mr. Itaru Yazawa, Imtakt Co. Ltd., for his kind supply of Cadenza TC-C18 and RP for protein columns.

Received for review July 23, 2003. Accepted November 20, 2003.

AC034840l