Metal-Containing Components in Medicinal Plants. II. Convenient Identification of Some Datura Species by Peptide Mapping of Their Ferredoxins

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A new method for plant identification is proposed in which each ferredoxin (Fd) is isolated from small amounts of fresh leaves of five species of genus Datura and the tryptic digest of each 5-carboxymethylated Fd is subsequently compared by peptide mapping using reversed-phase high-performance liquid chromatography (HPLC). This method makes it possible to analyze five samples in 4 or 5 d. The results obtained for the five plants indicated the HPLC patterns which reflect amino acid sequences of Fd to possibly be correlated to their morphological similarity.

Keywords D. metel (D. alba); D. inoxia; D. stramonium var. stramonium; D. stramonium var. tatula; D. arborea; Datura species; ferredoxin; peptide mapping; identification; high-performance liquid chromatography

In pharmacognosy it is important to identify closely related medicinal plants scientifically. The most reliable method is to analyze whole genes from each plant; however, even at the present time, this is too difficult. It may be possible to identify such plants by the amino acid sequences of their common proteins that reflect the nucleotide base sequences of the corresponding parts of the genes. In the course of our studies to elucidate metal-containing components of medicinal plants, we found that iron in the anionic soluble fraction from Datura species is present mainly as ferredoxin (Fd), one of the iron–sulfur proteins. Amino acid analysis for each Fd indicated slight differences in amino acid composition among them, suggesting the possibility that the amino acid composition or sequence of the protein provides valuable information for identification of Datura species.

In this study, to establish a rapid identification method for Datura plants by differences in amino acid sequences of Datura Fds, we minimized the scale of starting leaves and purified Fds rapidly by a mini-column, fast protein liquid chromatography (FPLC), and high performance gel chromatography. Peptide mapping by reversed-phase high performance liquid chromatography (RP-HPLC) was used to detect differences in amino acid sequences. By this method, it is possible to analyze five Datura plant samples in 4 or 5 d. The results may provide valuable information about the taxonomy of Datura species.

Experimental

Apparatus Isolation and analyses for Fds and its related substances were carried out on a Waters HPLC system (model 510 pumps, M680 gradient controller, and M441 absorbance detector) equipped with a stainless steel column, μ-Bondasphere C18-100 Å (3.9 mm i.d. x 15 cm) (Waters Assoc.). The purification of Fds was performed on a Waters W650 protein system using Mono Q column (HR5/5, Pharmacia).

Materials Datura metel L. (D. alba Nees) (chousen asagao in Japanese), D. inoxia Mill. (kochousen asagao), D. stramonium var. stramonium (shirobana youshu chousen asagao), and D. stramonium var. tatula (youshu chousen asagao) were cultivated in a greenhouse at this university. D. arborea L. (kodachi chousen asagao) were cultivated in an herb garden at this university. D. metel (D. alba) has white corollas, glabrous stem and leaves, and very short spines or tubercle on the capsules. D. inoxia has white corollas, leaves and stem covered with soft hairs, and capsules covered with long, slender spines. D. stramonium var. stramonium has white corollas, ovoid and spiny capsules, glabrous green stem, and glabrous, ovata and sinuately dentate leaves. D. stramonium var. tatula has lavender corollas and purple stem. The other features are the same as those of D. stramonium var. tatula. D. arborea, the tree Datura, has white large corollas, woody stem, and long and slender capsules.

Recommended Procedures Isolation of Fds from Each Type of Datura Leaves: Homogenize the frozen fresh Datura leaves (10 g) in an ice bath with 20 ml of 0.02 M Tris-HCl buffer, pH 7.5, and filter the homogenate through two layers of cloth mesh. After centrifugation at 16000 x g, apply the supernatant onto a mini-column (7 mm i.d. x 6 cm, Muromachi Kagaku) containing 2.5 ml of diethylaminomethyl (DEAE) cellulose (DE52, Whatman), equilibrated with 10 ml of 1 M Tris-HCl buffer, pH 7.5, followed by 10 ml of 0.02 M Tris-HCl buffer. Wash the column with the buffer containing 0.2 M NaCl (10 ml) and then elute the desired fraction with the same buffer containing 0.4 M NaCl (10 ml). Dilute the eluent (10 ml) to 25 ml with the NaCl free buffer, and apply the solution onto another mini-column containing 1 ml of the pre-equilibrated DEAE cellulose, followed by elution of 1 ml of the buffer containing 1 M NaCl. The desired portion is recovered in the 1 ml. Inject the desalted solution by gel filtration (NAP-10 column, Pharmacia) into the Mono Q column (gradient of 0–0.75 M NaCl in 0.02 M Tris-HCl), and collect the Fd fraction shown as the sharp peak at ca. 38 min (retention time, t0) in the case of the ultraviolet (UV) (420 nm) detection (Fig. 1). After concentrating this fraction to ca. 100 µl by ultrafiltration (Centricon 10, Amicon), inject ca. 100 µl of the solution into the Protein-pack 125 column (mobile phase: the buffer containing 0.2 M NaCl). Pool the reddish fraction at 15 min (t11) (see Fig. 2), and concentrate the solution to about 50 µl by Centricon 10. The resulting solution is almost pure Fd.

Preparation for APO-Fd and S-Carboxymethylated (CM-) Fd: Transfer the almost pure Fd solution (50 µl) to a microtube (Eppendorf tube, 1.5 ml). Add 1.8 M trichloroacetic acid (TCA) (25 µl) to the solution, collect the precipitate by centrifugation, and wash it with 20 µl of 0.6 M TCA by centrifugation. Carboxymethylation of the obtained apo-Fd is performed according to a slightly modified method of Crestfield et al. to Dissolve the precipitate in 100 µl of 0.4 M Tris-HCl buffer, pH 8.6, containing 6 µg guanidine HCl and 0.2% ethylenediaminetetraacetic acid (EDTA), and add 4 µl of β-mercaptoethanol. Allow to stand for 3 h in an airtight condition, and add 10.7 µg of iodoacetic acid (IAA) in 40 µl of 1 M NaOH. Again allow the solution to stand for 15 min after the addition of 25 µl of 1 M NaOH to adjust the pH to neutral. Change the buffer containing IAA using NAP-10 to 0.02 M Tris-HCl, pH 7.5. All steps after the addition of IAA should be conducted without exposure to light to prevent sub-reaction. Next, isolate pure Fd by RP-HPLC (μ-Bondasphere C18, trifluoroacetic acid (TFA)/CH3CN/water solvent system [A = 0.1% TFA; B = 0.1% TFA, 100% CH3CN] with a gradient program [23% B–48% B in 24 min; flow rate 1 ml/min]. The main peak (500 µl) at 16 min (t0) is collected into a microtube, change the solvent to 0.1 M NH4HCO3 (pH 8.5) for trypsin digestion.

Trypsin Digestion and Comparison of HPLC Patterns: Add 100 µg of trypsin (40 µg) to the buffer solution containing CM-Fd and allow it to react at 37°C for 3 h. Add 10% TFA solution (35 µl) to 700 µl of digestion solution, and inject into the reversed-phase column (μ-Bondasphere C18, the same solvent system as used in the isolation of CM-Fd with the gradient program [%B = 40% B in 50 min], flow rate 1 ml/min). The obtained chromatograms are then compared with each other.

Results and Discussion

To establish a routine method to determine the differences
in the amino acid sequence of Fd, attempts were made to scale down the amount of starting material (leaves) to only 10 g and simplify the procedure. In previous experiments, since about 1 kg of _Datura_ leaves was used as starting material, comparatively large amounts of resin such as DEAE cellulose and Sephadex G-75 and preparative-size columns were required. In this study, a mini-column, FPLC and HPLC were primarily used for rapid analysis. First, a mini-column containing 2.5 ml of DEAE cellulose was used for the adsorption of Fd. The adsorbed Fd was eluted with the 0.02 M Tris-buffer, pH 7.5, containing 0.4 M NaCl, whereas no Fd was eluted with the buffer containing 0.2 M NaCl. Consequently, the Fd fraction adsorbed from _Datura_ leaves was eluted from the mini-column with the buffer containing 0.4 M NaCl after washing the column with the buffer containing 0.2 M NaCl to remove extraneous substances. A crude Fd fraction (1.5 ml) was obtained by concentrating the resulting solution (10 ml) using another mini-column containing 1 ml of DEAE cellulose and desalting by gel filtration (NAP-10).

Figure 1 shows the FPLC chromatograms of the crude Fd fractions eluted with 0.4 M NaCl buffer. Even at this step, Fd appeared as a sharp peak at about 40 min \( t_r \). The pool of the peak was further purified by high performance gel chromatography (Protein-pack 125, Waters) (Fig. 2). The peak at 17 min \( t_r \) is pure Fd. After treatment of TCA followed by carboxymethylation, Cm-Fd was finally purified by RP-HPLC as seen in Fig. 3. Note that four Cm-Fds from _metel_ (alba), _innoxia_, _stramonium_, and _stramonium_ var. _tutula_ have their main peaks at the same retention time (15.0 min), whereas the main peak of _D. arborea_ Cm-Fd appeared at a different retention time (15.6 min). Indeed, the mixture of _stramonium_ var. _tutula_ and _arborea_ Cm-Fds exhibited two peaks (the second arising from _arborea_ Cm-Fd). This suggests that the primary structure of _arborea_ Fd is somewhat different from those of other _Datura_ Fds.

Figure 4 shows HPLC chromatograms of peptide
mixtures of tryptic digestion from each *Datura* Fd. These are thought to be more sensitive to differences in the amino acid sequence of Fd than those for the whole Cm-Fd. *D. metel* (*D. alba*) and *innoxia* gave very close chromatograms in which only the ratios of peak heights slightly differed. Thus, the chromatograms were virtually identical. *D. stramonium* var. *stramonium* and var. *tatula* gave identical chromatograms. Their peak patterns at around 45 min (tₚ) differed from those for *D. metel* (*D. alba*) and *innoxia*. One clear distinction is the two peaks at 27 min (tₚ) observed only in the former group (Fig. 4). In our previous report, amino acid analyses on four *Datura* Fds indicated *metel* (*alba*) and *innoxia* to possess two arginine residues in each molecule, whereas *stramonium* var. *stramonium* and *stramonium* var. *tatula*, only one. Since trypsin hydrolyzes the peptide bond at the carboxyl side of arginine and lysine residue, the arginine-containing long peptide may possibly be degraded by this enzyme to form the two additional shorter peptides which appear as the peaks at 27 min (tₚ). However, the corresponding long peptide must remain unchanged in *stramonium* and *tatula* Fds. The chromatogram of the peptide mixture from *arborea* Fd, having main peaks at 44.8 and 46.0 min (tₚ), is apparently distinguished from those of any groups (42.4 and 44.0 min for the *alba* group; 43.2, 44.0, and 45.6 min for the *stramonium* group). Of interest is the fact that morphologically analogous plants, *D. metel* (*D. alba*) and *D. innoxia*, or *D. stramonium* var. *stramonium* and *D. stramonium* var. *tatula*, exhibit the same HPLC patterns, although three groups belonging to different sections in genus *Datura* give distinct patterns. *D. metel* (*D. alba*) and *D. innoxia*, *D. stramonium* var. *stramonium* and *D. stramonium* var. *tatula*, and *D. arborea* belong to the *Datura* section, *Stramonium* section, and *Brugmansia* section, respectively. The latter is thought to differ considerably from other sections of the genus *Datura*, the so-called herbaceous *Datura*. In practice, the species forming the section *Brugmansis* were first placed in a distinct genus by Person in 1805. This seems consistent with the finding that *D. arborea* Fd exhibits a chromatogram distinct from others, even in HPLC of Cm-Fd.

Some confusion still remains in naming of the two varieties of *D. stramonium*, *D. stramonium* with white flowers and *D. tatula* with purple flowers, both having spiny capsules, were proposed by Linnaeus in 1753 and 1762, respectively, as two distinct species. Other botanists, however, were inclined to regard them rather as varieties. The breeding experiments of several botanists have proved that white-flowered *D. stramonium* L. and the purple-flowered *D. tatula* L. differ only by a single pair of genes and that they belong to a single species. In addition, the breeding experiments of Tabata *et al.* on morphological characters and alkaloid content support the proposal by Blakeslee and others that *D. stramonium* L. and *D. tatula* L. should be considered two varieties of a single species.

So, *D. stramonium* var. *stramonium* and *D. stramonium* var. *tatula* are used here, as many present botanists do. In this connection, it is of interest that these two *Datura* plants gave reasonably the same chromatograms in this study. More detailed study on the taxonomy of *Datura* species should be advanced by careful determination of the primary structures of each Fds.

In conclusion, this method, in which each Fd is isolated from a small amount of plant leaves and the HPLC patterns of the tryptic digest of Cm-Fds are subsequently compared to determine whether amino acid sequences are identical, should be found useful for identifying closely related plants scientifically.

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References