Amino Acid Sequences of Ferredoxins from *Atropa belladonna* and *Hyoscyamus niger*: Their Similarities to Those in Other Tropane-Alkaloid-Containing Plants

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The complete amino acid sequences of [2Fe–2S] ferredoxin from *Atropa belladonna* and *Hyoscyamus niger* have been determined by automated Edman degradation of the entire S-carboxymethyl cysteinyl proteins and of the peptides obtained by enzymatic digestion. These two ferredoxins exhibited 1—8 differences in their amino acid sequences compared to those of other tropane-alkaloid-containing plants (*Scopolia japonica*, *Datura stramonium*, *D. metel*, and *D. arborea*), and only 1 or 4 differences compared to *S. japonica* and *D. arborea*. In contrast, 9—23 differences were observed among the other solanaceous ferredoxins. This suggests that tropane-alkaloid-containing plants are closely related taxonomically.

Key words *Atropa belladonna; Hyoscyamus niger; Solanaceae; amino acid sequence; protein chemotaxonomy; ferredoxin*

We have proposed the term ‘protein chemotaxonomy’ for molecular taxonomy based on the primary structures of common plant proteins, instead of the so-called secondary metabolites. To evaluate the effectiveness of this concept, we have carried out a series of studies on the family Solanaceae, using ferredoxin (Fd), an iron–sulfur electron-transfer protein.2) This protein was chosen for this study because it is easy to isolate and has an appropriate molecular weight for determining the primary structure. Previously, we have reported the primary structures of Fds from seven *Datura* plants3—6) and other solanaceous plants7—11) Our recent results have suggested that *S. japonica* is closely related taxonomically to *Datura* plants, and especially to *D. arborea*. Interestingly, both *S. japonica* and *Datura* plants contain tropane-alkaloids such as hyoscyamine, atropine, and scopolamine. It may be worthwhile to determine the relationship between pharmacologically important constituents and the amino acid sequences of Fds from these plants. These considerations led us to elucidate the amino acid sequences of Fds from *Atropa belladonna* and *Hyoscyamus niger*, which are important medicinal plants that contain tropane-alkaloids.

In this report, we determined the primary structures of Fds from *A. belladonna* and *H. niger* (Solanaceae), and compared them with those of Fds from other higher plants as well as those from *Datura* plants and other solanaceous plants.

MATERIALS AND METHODS

**Materials** *Atropa belladonna* and *Hyoscyamus niger* were cultivated in the herb garden at Osaka University of Pharmaceutical Sciences.

**Isolation of Ferredoxin** Each protein (15.4 or 3.0 mg) was purified from the fresh leaves (0.6 or 0.5 kg) of *A. belladonna* or *H. niger* as described previously.3,7)

**Sequence Determination** The amino acid sequences of the Fds were determined using a gas-phase protein sequencer with automated Edman degradation of 5-carboxymethylcysteiny (Cm) Fd and the peptides obtained by lysyl endopeptidase, trypsin, or endoproteinase Asp-N digestion. C-terminal analysis was carried out with carboxypeptidase Y.

The detailed procedure and the other methods have been described previously.3,7)

**Construction of a Phylogenetic Tree** A phylogenetic tree was constructed from the amino acid sequences (97 residues) of higher-plant Fds (30 species) using the unweighed pair-group method with the arithmetic averages (UPGMA) method of Nei (GENETYX software, Software Development, Japan).12)

RESULTS AND DISCUSSION

**Properties** The absorption maxima in the UV–Vis spectrum of *A. belladonna* (*Ab*)-Fd were at 275, 285 (sh), 330, 420, 465 nm, and showed *A*max/*A*275 nm ratios of 0.83, 0.63, and 0.57, respectively. *H. niger* (*Hn*)-Fd exhibited virtually the same spectrum as *Ab*-Fd. These spectra were characteristic of [2Fe–2S] Fds from other higher plants.1,3) The molar absorption coefficient at 420 nm, based on the spectrum and protein determination, was 11000 M–1 cm–1, which was similar to those of other higher-plant Fds.2,13) The biological activities and other physico-chemical properties of *Ab*- and *Hn*-Fds will be published elsewhere, together with those of other solanaceous Fds.

**Sequence Determination** The sequencing strategy is summarized in Fig. 1. The analytical results regarding the amino acid compositions of both Cm-Fds and the peptides obtained by enzymatic digestion, were consistent with the derived sequences. Automated Edman degradation of the *Ab*-Cm-Fd yielded the amino-terminal sequence up to the 42nd cycle. Lysyl endopeptidase digestion gave two short peptides [L-1 (1—4) and L-2 (5—6)] and two long peptides [L-3 (7—50) and L-4 (51—97)]. As in many other Fds, Lys-91 was insensitive to lysyl endopeptidase cleavage due to the adjacent sequence Lys–Glu–Glu–Glu–. These peptides were isolated by reversed-phase HPLC; their *Rt* values were 15.2 for L-1, 44.8 for L-3, and 45.8 min for L-4,5, while L-2 was missing. Edman degradation of L-3-T-2, obtained by trypsin digestion of L-3 (7—50), confirmed the sequence of 41—50. L-4,5 covered the sequences of 50—97 (C-terminal), except for several dubious amino acid residues of the end part. Se-
sequence analysis of L-4,5-D-6 (84—97), obtained by endo-
proteinase Asp-N digestion of L-4,5, confirmed the end part 
of L-4,5 (91—97). The N-terminal sequence was confirmed 
by the isolation of L-1 (Ala–Thr–Tyr–Lys). In addition, car-
boxypeptidase Y digestion of the Cm-Fd for different periods 
of time suggested that the C-terminal sequence was –Leu– 
Thr–Gly–COOH. This result was in reasonable agreement 
with the C-terminal sequence obtained by Edman degrada-
tion of the peptide, L-4,5-D-6 (84—97).

The sequence analysis for 
Hn-Fd was conducted in the 
same manner except that additional peptides, L*-3 (7—50) 
and L*-4,5 (53—97), were obtained because of the coexis-
tence of a mutated minor Fd. In HPLC, L*-3 (7—50) 
from the minor Fd was eluted at 45.2 min just after L-3 (7—50) 
(t$_R$/H11005 44.8 min) and L*-4,5 (53—97) from the minor Fd was 
eluted at 46.0 min just before L-4,5 (51—97) (t$_R$/H11005 46.8 min).
The sequence analysis clarified that Asn-17, Ser-52, and Ile-
69 were mutated to Asp-17, Lys-52, and Met-69 in the minor 
Fd, respectively.

These results led to the complete amino acid sequences 
shown in Fig. 1 for Ab- and Hn-(major and minor)-Fds. Their 
primary amino acid sequences are compared with those of solana-
ceous Fds in Fig. 2.3—6,9—11) Compared with other solana-
ceous Fds, differences were observed at Ser-52 in both Fds, 
as with Spj- and Lc-Fds, and at Lys-52 in Hn-(minor)-Fd, in 
addition to 32 positions, namely 2, 8, 11, 13—15, 17, 21, 22, 
27, 31, 34, 43, 51—53, 55, 59, 61—63, 69—71, 73, 82, 84, 86, 
93, 94, 96, and 97, from the amino-terminus, where the 
residue Lys-52 in Hn-(minor)-Fd is only observed in the primary 
structures of Fds from Spinacia oleracea (Chenopodiaceae) 
and Colocasia esculenta (Araceae). The residue Ser-52 in solana-
ceous Fds, as in Spj- and Lc-Fds, is rare in the sequences of other higher plants. This residue is 
characteristic of Ab-, Hn-, Spj-, and Lc-Fds. Also, the residue 
His-33 may be characteristic of solanaceous Fds. In Fds, the 
sequence 35—50, including the sequences –C39–C44–C47– 
which participate in chelation to iron atoms, the sequence 
74—77, which contains the last cysteine ligand (–C77–) for 
the iron atom, and the later region, 83—93, are almost per-
fectedly conserved. This was also true in the case of Ab- and 
Hn-Fd.

**Taxonomic Considerations** Although some 3500 
species are known to be members of the Solanaceae, the 
arrangement of the species into genera and other useful 
groups is still not clear.14) The genus Atropa, containing 
about 4 species, is represented by A. belladonna. The genus 
Hyoscyamus containing about 20 species, is represented by 
H. niger. Both A. belladonna and H. niger, dealt with here, 
are important medicinal plants that contain tropane-alkaloids 
such as hyoscyamine, atropine, and scopolamine. The gen-
era, *Atropa* and *Hyoscyamus*, belong to different subtribes, 
Lyciinae and Hyoscyaminae, respectively, of the same tribe, 
Solanaceae.

Many primary structures have been reported for chloro-
plast [2Fe–2S] Fds.3—6,9) The number of amino acid 
differences are 14—40 for different families and 0 to 4 for the 
same genus.3—6,9) Although there only limited data are 
available concerning plants that belong to the same family, 
the sequences of several solanaceous Fds have been deter-

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**Fig. 1. Amino Acid Sequences of *Atropa belladonna* and *Hyoscyamus niger* (Major and Minor) Ferredoxins**

Arrows (→) and (←) represent residues determined by automated Edman degradation and carboxypeptidase Y digestion, respectively. L (1—5), T (1—2), and D (1—6) represent peptides obtained from lysyl endopeptidase, trypsin, and endoproteinase Asp-N digestion, respectively. * In Hn-(minor)-Fd, D*-5 (26—56) and L*-4,5 (53—97) were sequenced. Different amino acids are indicated by white on black.
mined. Table 1 shows a matrix of amino acid differences in solanaceous Fds that have been determined so far. Two to nineteenth amino acid differences were observed among different genera, *Datura*, *Physalis*, *Nicotiana*, *Capsicum*, *Scopolia*, and *Lycium*. On the other hand, there were only 0—4 differences in the Fds from seven *Datura* plants. In our recent study, large differences (12—19) in the amino acid sequence were observed among the four *Solanum*-Fds, suggesting that these *Solanum* plants are quite remotely related. In the present study, only three differences in the amino acid sequence were observed between *Ab*- and *Hn*-Fds, suggesting that *A. belladonna* and *H. niger*, which belong to different subtribes, Lyciinae and Hyoscyaminae respectively, are quite closely related. Interestingly, only 1—8 differences were observed among *Ab*- and *Hn*-Fds, *Sj*-Fds, and *Datura*-Fds; *Ab*-Fd showed 1, 5, 4, 10, 9, 16, 2, 1, 9, 20, 18, and 4 differences, and *Hn*-Fd showed 4, 8, 5, 12, 12, 18, 24, 27, 25, 26, 24, 25, 25, 25, 25, 25, 25, 25, and 25 differences respectively, compared to the Fds of *D. arborea*, *D. metel*, *D. stramonium*, *P. alkekengi* var. *Francheti*, *N. tabacum*, *C. annuum*, *L. chinense*, *S. japonica*, *S. abutiloides*, *S. indicum*, *S. August 2005 1537

Fig. 2. Comparison of the Sequences of [2Fe–2S] Ferredoxins from Solanaceous Plants

Amino acids are represented by one-letter abbreviations.

Table 1. Amino Acid Difference Matrix of [2Fe–2S] Ferredoxin Sequences for Higher Plants

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a) Data among the higher plants except for solanaceous plants are available in ref. 7 and are therefore omitted here. See legend to Fig. 2. (1)—(15) belong to Solanaceae; (16) to Cruciferae; (17) and (18) to Leguminosae; (19) to Umbelliferae; (20) and (21) to Phytolaccaceae; (22) to Chenopodiaceae; (23) to Caprifoliaceae; (24) to Compositae; (25) to Araceae; (26)—(28) to Graminaceae; (29) and (30) to Equisetales; and (31) to Filicales, respectively.
lyratum, and S. nigrum. One to eight differences among Ab-, Hn-, Sj-, Lc-, and Datura-Fds were significantly smaller than those (9—23) among the solanaceous Fds except for Ab-, Hn-, Sn-, Sj-, Lc-, and Datura-Fds.

Figure 3 shows the phylogenetic tree based on the Fd sequences of higher plants. 12) Fifteen solanaceous plants [containing Hn (minor)-Fd] form a cluster that is distinctly separated from other angiospermous plants, ferns, and horsetails by appreciable long branch lengths, which increase in that order. In the solanaceous cluster, five genera, Atropa, Hyoscyamus, Scopolia, Lycium, and Datura, are separated from each other by short branch lengths, which suggests a close taxonomic relationship among them. 18) In fact, the genera Atropa and Lycium belong to subtribe Lyciinae, and the genera Hyoscyamus and Scopolia belong to subtribe Hyoscyaminae of the same tribe Solaneae, respectively. However, a conflicting result was also obtained. These genera form one cluster together with three species of the genus Datura, especially D. arborea with a very short branch length, although the genus Datura belongs to the tribe Datureae rather than Solaneae. These results suggest that the genus Datura is in a close taxonomic relation to the genera Atropa, Hyoscyamus, Lycium, and Scopolia. In this regard it is of special interest that A. belladonna, H. niger, and S. japonica as well as Datura plants contain tropane-alkaloids.

In conclusion, comparison of the amino acid sequence of Fd indicated that there are very close relationships among plants that contain tropane alkaloids, which are characteristic constituents of solanaceous plants.

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REFERENCES AND NOTES

18) In this solanaceous cluster, four Solanum plants are located among many genera, indicating a discrepancy between protein chemotaxonomy and classical taxonomy. This result suggests that these Solanum plants are distantly related to each other and that genus Solanum includes a very wide variety of plants. This view is consistent with the fact that Solanum is a huge genus that contains over 1000 of the total number of solanaceous plants.