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**A novel membrane protein in spinach chloroplast and its homologue in  
*Arabidopsis thaliana***

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**Introduction**

Two-dimensional gel electrophoresis (2-D) of proteins combining with microsequencing analysis provides a powerful approach for the identification of thylakoid membrane proteins and the N-terminal sequence information for cloning of corresponding genes [O'Farrell 1975, Celis and Bravo (Eds.) 1984, Yu et al 1994a, Yu et al 1994b, Yu and Björn 1997]. In order to purify and to characterize protein spots in the basic range of 2-D (i.e.  $I_p > 7.00$ ), we have modified the non-equilibrium pH-gradient electrophoresis (NEPHGE) gel system and adapted it to the protein analysis of chloroplast membranes. In the present communication, it is reported that on the basis of microsequence data and the sequence comparison through databases, a protein having a molecular size of 40 kDa and focusing at pH range of 7.8 (named as Neph-40) is a novel one and the sequence of its homologue in *Arabidopsis thaliana* is presented.

**Materials and Methods**

Spinach plants (*Spinacia oleracea*) were cultivated at 18°C under fluorescent light with 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation and a light/dark cycle of 12 h. Thylakoids were prepared from spinach leaves. The preparative procedure was as described previously [Albertsson and Yu, 1988]. The membrane vesicles derived from either the grana-lamellae or the stroma-lemellae of the thylakoids were isolated according to the description by Yu et al [1994a]. The solubilization and the isolation of thylakoid membrane proteins were performed following the procedure of our previous description [Yu et al., 1994a]. 2-D Gel electrophoresis was carried out in such a way that non-equilibrium pH-gradient electrophoresis (NEPHGE) in the first dimension replaced the IEF (isoelectric focusing) which was used in the previous work [Yu et al 1994a], while the second dimension of the gel electrophoresis was performed in the same way as before [Yu et al 1994a]. The gel mixture of the NEPHGE contains urea (4.12 g), acrylamide (0.975 ml of a stock solution containing 28.38% acrylamide and 1.62% bisacrylamide), 10% NP40 (1.5 ml), H<sub>2</sub>O (1.5 ml), Ampholine pH range 7-9 (0.225 ml, Pharmacia Biotech AB, Uppsala, Sweden. Code No.80-1125-94),

Ampholine pH range 8-10.5 (0.150 ml, Pharmacia Biotech AB, Uppsala, Sweden. Code No.17-0455-01), ammonium persulphate (15  $\mu$ l) and TEMED (N,N,N',N'-tetramethylethylenediamine) (10.5  $\mu$ l, Sigma, USA) in a total volume of 7.5 ml, which then was distributed into rod-shaped glass tubes with 2 mm diameter. The NEPHGE was run directly at 400 volts for 4.5 h at room temperature without the prerun step. The essential part of this procedure is to exchange the running buffer between the upper and the lower chambers, i.e., the upper chamber should be filled with 10 mM H<sub>3</sub>PO<sub>4</sub> and the lower chamber with 20 mM NaOH. The other steps for the 2-D electrophoresis were the same as described previously [Yu et al., 1994a]. The detailed procedures for the purification of polypeptide spots and microsequencing were described earlier [Yu et al 1994a]. The EMBL updated version 6 and the swissprot databases, as well as CD-Search using BLAST program were used for searching the homology sequences. The GCG software (from UWBC) was used for the sequence analyses.

## **Results and Discussion**

### *[1] 2-D protein map of spinach thylakoid membrane*

It has previously been reported that there are about 100 polypeptides resolved by two-dimensional gel electrophoresis with IEF as first and SDS-PAGE as second dimensions and 18 polypeptide spots in the protein pattern of thylakoids have already been identified by microsequencing and immunostaining [Yu et al, 1994a]. In the present work, the thylakoid membrane proteins are displayed by using the NEPHGE system. About 50 polypeptide spots are shown in the gel pattern and five of them have been identified by microsequencing and database-searching. Their N-terminal amino acid sequences determined in this work are shown in Table 1. Database searching reveals that four of these five polypeptides (No.2-No.5) are homologous to the known proteins and details are: No.2 is ferredoxin-NADP-oxidoreductase which can be also seen in the previous 2-D protein pattern [Yu et al, 1994a] due to overlap between these two kind of gels, the No.3 is PsaD polypeptide of PSI complex, No.4 is the 16 kDa oxygen evolving protein and No.5 is the PsaE polypeptide of PSI complex. Combining these two kinds of 2-D, almost a full range of the 2-D protein pattern involving isoelectric focusing from acidic to basic pH for the spinach thylakoids is obtained. It displays about 150 membrane polypeptides and to our knowledge, only about a half of this number of polypeptides in the thylakoids have been identified and characterized so far. The remaining thylakoid polypeptides distributed in the 2-D gel need also to be clarified with respect to their identities and properties in the process of photosynthesis. The 2-D electrophoresis together with microsequencing analyses provides a powerful and convenient approach for this purpose.

### *[2] Characterization of a novel thylakoid membrane protein (Neph-40)*

A polypeptide having a molecular size of 40 kDa and focusing at pH range of 7.8 in NEPHGE of spinach chloroplast is a novel one, based on the database-search with its N-terminal sequence. This polypeptide is therefore named as Neph-40 (No.1 in Table 1).

**Table 1.** N-terminal sequences of five polypeptides of spinach thylakoids resolved by 2-D electrophoresis involving NEPHGE in the first dimension and SDS-PAGE in the second dimension, and determined by the transblotting and microsequencing technique. The numeral of each sequence is corresponding to polypeptide spot having the same one demonstrated in Fig 3-4.

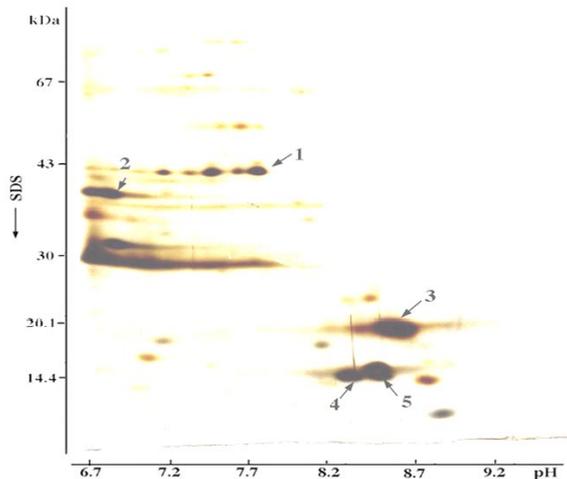
- No.1: VSLPKEQLVTSLSLQVEQT;  
 No.2: QIASDVEAPPC;  
 No.3: AAAAEGKAAAATETKEAPKG;  
 No.4: EARPIVVGP-PCLSGGLPGTE;  
 No.5: AEEAAAAPPAASPEGEAPLAE.

It turns out that Neph-40 has 66.7% identity and 89% similarity with the N-terminal region of a protein sequence deduced from EST (Expressed Sequence Tag) (Accession No: T46513) and from the DNA sequence of the chromosome 5 from *Arabidopsis thaliana* (Accession No: AB006708) (Fig.1). The open reading frame of the genomic DNA sequence in *Arabidopsis thaliana* is 1652 bp long and contains 6 exons and 5 introns. The coding sequence is 1161 bp encoding 387 amino acid residues (Fig. 2). The molecular mass estimated from the amino acid sequence of this protein is also about 40 kDa which fits well with Neph-40. The start amino acid residue, Val, of the N-terminal sequence of the novel polypeptide is corresponding to the residue position 59 of its homologue in *Arabidopsis*. The amino acid sequence 1-58 is therefore suggested to be a signal peptide, which would lead this nuclei-encoded protein into chloroplast. The functional role of Neph-40 in the process of photosynthesis is not clear. However, since its homologue in *Arabidopsis thaliana* contains a Rhodanese homology domain which has been found in some enzymes, for example, protein phosphatase, sulfide dehydrogenase, etc., we therefore suggest that Neph-40 may play a role in redox reactions of photosynthesis.

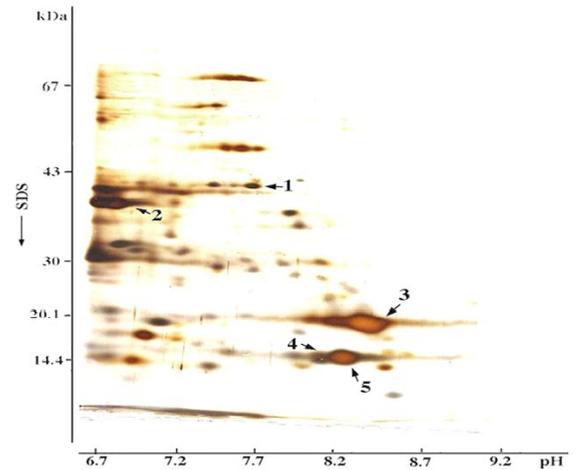
	Spinach Neph-40:	1	VSLPKEQLVTSLSLQVEQT	18
			:     :   :   .       :     .	
	<i>Arabidopsis thaliana:</i>	59	VSIPKDQIVSSLTEVEKT	76
1	ATGGCTATGGCGGAAATGGCAACGAAGTCTTCACTATCTGCAAACTCACTCTTCTTCTTCTTCTACTAAGAAGACTGAGTCTGAGA			
90	M A M A E M A T K S S L S A K L T L P S S S T K K T L S L R			
91	CAAGTCTCTGTTTCACTTCCAACATCAACTTCAATCTCTCTGTTATCTCTCTTTGCATCTCCTCCTCATGAAGCTAAAGCTGCTGTTTCC			
180	Q V S V S L P T S T S I S L L S L F A S P P H E A K A A <u>V S</u>			
			↓	
181	ATTCCAAGGACCAAATCGTCTCCTCTCTCACTGAAGTGGAGAAAACAATCAACCAAGTTCAAGAACTGGTCTAGTGTATTTGATGCA			
270	<u>I P K D Q I V S S L T E V E K T</u> I N Q V Q E T G S S V F D A			
271	ACGCAGCGTGTGTTCCAAGTAGTAGGAGATGCTCTTAAACCAGCTTTGGACTGCTTTACCCATTGCAAAGCAAGCTGGTGAAGAAGCT			
360	T Q R V F Q V V G D A L K P A L D T A L P I A K Q A G E E A			
361	ATGAAGCTTGCTTCTCCTGCTTCTCTGAAGCTTCAAAGAAAGCTCAAGAAGCAATGCAGAGCTCTGGTTTTGATTCTGAGCCTGTCTTT			
450	M K L A S P A F S E A S K K A Q E A M Q S S G F D S E P V F			
			↓	
451	AATGCTGCAAAGACAGTAACAGATGTAGCACAAACAGACGTCAAAGCGATAGAAGATGCTAAACCGATTGCTTCATCGACCATGGATACG			
540	N A A K T V T D V A Q Q T S K A I E D A K P I A S S T M D T			
541	ATTTCTTCAGTGCACCTAGTGTCAATTGTTGTTGCTGCTGGTGTGCGTTTCTTGCTTACCTTCTTCTCCCTCTGTTTTCTCTGCCATC			
630	I S S A D P S V I V V A A G A A F L A Y L L L P P V F S A I			
			↓	
631	TCTTTAACTTCCGTGGTTCAAAGGTGATCTTACGCCGGCTCAAACGCTTGACCTTCTTTGCACCAAGAACTACTTGATGGTGGATATA			
720	S F N F R G Y K G D L T P A Q T L D L L C T K N Y L M V D I			

### [3] Distribution of Neph-40 in spinach chloroplast

Protein analysis for thylakoid fragments derived from different parts of the spinach thylakoids reveals that the Neph-40 is localized in both the stroma lamellae and the grana lamellae of chloroplast. Fig 3 and 4 demonstrate its distribution (No.1). The secondary structure analysis of this protein confirms that Neph-40 has typical membrane spanning domains (data is not shown).



**Fig. 3.** 2-D protein pattern of the NEPHGE system for the fraction derived from grana lamellae of the thylakoids. Total 100 µg of the polypeptides solubilized in the lysis buffer were loaded into the NEPHGE system. Silver staining was applied in order to get high resolution. Numerals represent identified polypeptides.



**Fig. 4.** 2-D protein pattern of the NEPHGE system for the fraction derived from stroma lamellae of the thylakoids. The numerals have the same meaning as those in Figure 3.

### Reference

- Albertsson P-Å, Yu S-G (1988) Heterogeneity among Photosystem II $\alpha$ . Isolation of thylakoid membrane vesicles with different functional antennae size of Photosystem II $\alpha$ . *Biochimica Biophysica Acta* **936**, 215-221. 90-94.
- Celis JE, Bravo R (Eds.) (1984) Two-Dimensional Electrophoresis of Proteins: Methods and Applications, Academic Press, New York.
- O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem* **250**, 4007-4021.
- Yu S-G, Stefánsson H, Romanowska E, Albertsson P-Å (1994a) Two dimensional electrophoresis of thylakoid-membrane proteins and its application to microsequencing. *Photosyn. Res.* **41**, 475-486.
- Yu S-G, Romanowska E, Xue Z-T, Albertsson P-Å (1994b) Evidence for two different Rieske iron-sulfur proteins in the cytochrome bf complex of spinach chloroplast. *Biochim. Biophys. Acta* **1185** 239-242.
- Yu S-G, Björn LO (1997) Effects of UVB radiation on light-dependent and light-independent protein phosphorylation in thylakoids proteins. *Journal of Photochem and Photobiol B: Biology* **37** 212-218.