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MOLECULAR HETEROGENEITY OF FERREDOXIN-NADP$^+$ REDUCTASE FROM SPINACH LEAVES

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SUMMARY: Highly purified ferredoxin-NADP$^+$ reductase from spinach leaves showed at least eight different protein bands in the electrofocused gel. All of them were catalytically active and were adsorbed on a ferredoxin-Sepharose 4B affinity column. The N-terminal amino acid sequence of the main component species was analyzed by the automatic Edman degradation method. It was found that when the reductase was stored at 4 °C, new protein bands appeared in isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoreses, but the appearance of the bands was suppressed by the addition of a protease inhibitor, diisopropyl fluorophosphate. This indicates that the molecular heterogeneity of the reductase may result from the digestion with a protease present in spinach leaves.

Ferredoxin-NADP$^+$ reductase [Ferredoxin:NADP$^+$ oxidoreductase, EC 1.18.1.2] from spinach leaves was highly purified and crystallized as reported by Shin et al. (1). Keirns and Wang, however, first reported that in a highly purified reductase sample, three different molecular species were still noticeable by isoelectric focusing (2). Since then, the nature and the properties of multiple forms of the reductase have been repeatedly reported by various workers (3-8). Gozzer et al. (5) described that five molecular species of the reductase, which were isolated by the isoelectric focusing, showed the differences in the molecular weights and the amino acid compositions but no significant differences in kinetic properties. Shin and Oshino observed that two different molecular species were separated by chromatography on a DEAE-cellulose column and reported that the component of a molecular weight of 75,000 might be a dimer of the other component of a molecular weight of 33,000 (6, 8). There are at present many discrepancies concerning the properties of the multiple forms among the workers.

Abbreviations: DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate.

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In the present paper, we describe that even a crystallized reductase sample is separated into at least eight component enzymes by the isoelectric focusing. On the basis of electrophoretic patterns of the sample stored in the absence and presence of a protease inhibitor, we demonstrate that the molecular heterogeneity is ascribed to the contaminating protease activity.

MATERIALS AND METHODS

Materials: Carrier ampholite (Servalyt AG 5-6) was purchased from Serva Feinbiochemica, Heidelberg. Diisopropyl fluorophosphate (DFP) and 2(p-iodophenyl)-3-nitrophenyl-5-phenyltetrazolium chloride were purchased from Sigma Chemical Co., St. Louis and Wako Pure Chemical Industries, Ltd., Tokyo, respectively.

Ferredoxin-$NADP^+$ reductase was prepared from spinach leaves by the method similar to that described by Shin et al. (1). The reductase was further purified on a ferredoxin-Sepharose 4B affinity column prepared according to the method of Shin and Oshino (6). The concentration of the reductase was determined spectrophotometrically using a molar extinction coefficient of 10.4 mM^{-1} cm^{-1} at 458 nm (9).

Electrophoreses in Polyacrylamide Gels: Isoelectric focusing was carried out as described by Righetti and Drysdale (10) with slight modifications. Sodium dodecyl sulfate (SDS)-slab gel electrophoresis was performed essentially after Laemmli (11).

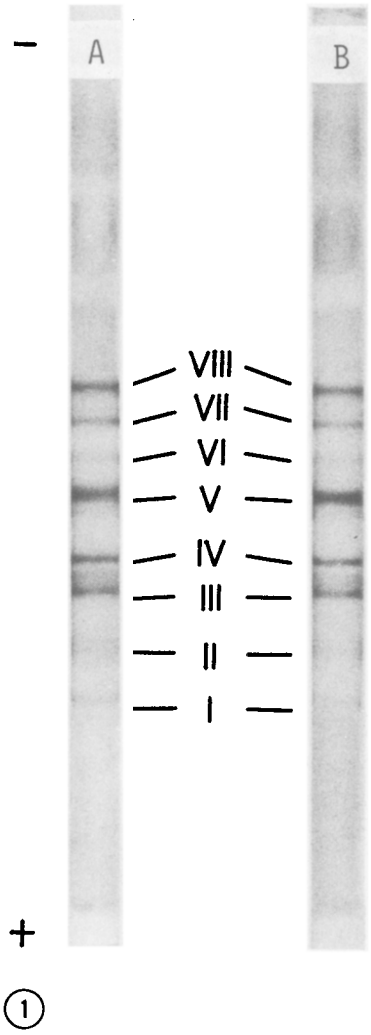
Staining Gels for Protein and Enzyme Activity: Proteins in the gel were stained with Coomassie Brilliant Blue G-250 (12). The enzyme activity of the bands was visualized by incubating the gel in 50 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM NADPH and 0.2 mM 2(p-iodophenyl)-3-nitrophenyl-5-phenyltetrazolium chloride. Gel scanning was performed at 600 nm (Coomassie Brilliant Blue) or 550 nm (enzyme activity) with a Shimadzu dual-wavelength TLC scanner, CS-910.

Isolation and Purification of Component Enzymes: The component enzymes electrofocused at 4 °C in a glass tube (7 x 150 mm) were extracted from the sliced gels with 50 mM Tris-HCl buffer (pH 7.4). After dialysis against 10 mM Tris-HCl buffer (pH 7.4), each of the component enzymes was subjected to the ferredoxin-Sepharose 4B affinity column and eluted with 10 mM Tris-HCl buffer (pH 7.4) containing 1 M NaCl.

Sequence Analysis: The carboxymethylated reductase was degraded with a Beckman spinning cup sequenator, model 890 C, using a quadrol single-cleavage program (a modification of the Beckman program, 122974). Phenylthiohydantoin derivatives were identified by thin-layer chromatography on Merck silica-gel plates (Kieselgel 60 F_{254}) using various solvent systems (13, 14). Histidyl and arginyl derivatives were identified by utilizing Pauly (15) and Sakaguchi (16) reactions, respectively.

RESULTS AND DISCUSSION

Highly purified ferredoxin-$NADP^+$ reductase, which had an absorbance ratio (A_{458}/A_{275}) of 0.13, was subjected to isoelectric focusing in polyacrylamide gel. As shown in Fig. 1A and Fig. 2 (solid line), at least eight different bands were noticeable by staining for protein. They are named as FNR-I to FNR-VIII in the order from anode to cathode. The eight different bands of the reductase had the isoelectric points of 5.26 ± 0.06, 5.41 ± 0.04, 5.57 ± 0.04, 5.68 ± 0.03, 5.88 ±



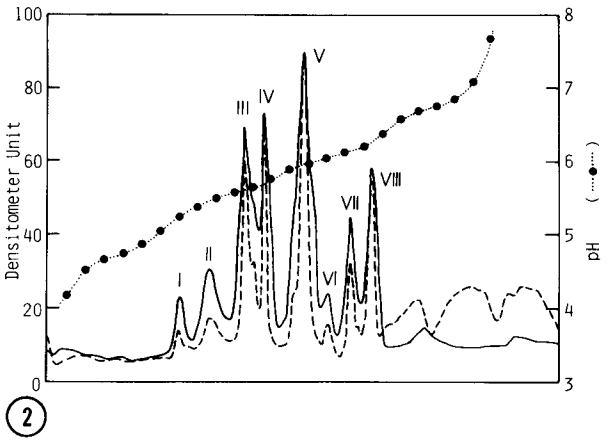


Fig. 1 Isoelectric focusing of ferredoxin-$NADP^+$ reductase in 7.5 % acrylamide gel containing 1 % carrier ampholytes with buffering capacity in the pH range of 5-6. Gel A was stained for protein and gel B for enzyme activity. The amount of the reductase applied to the gel was 100 µg.

Fig. 2 Densitometric scanning traces and pH gradient profile of the electrofocused gels shown in Fig. 1. Gel scannings were performed at 600 nm for protein (————) and at 550 nm for enzyme activity (– – – –), respectively.

0.07, 5.94 ± 0.07, 6.05 ± 0.08, and 6.21 ± 0.08, respectively. The relative amounts of these bands were calculated to be 5.7 ± 2.2, 10.3 ± 2.4, 17.3 ± 2.4, 9.8 ± 1.4, 21.0 ± 3.1, 4.6 ± 0.9, 7.4 ± 0.7, and 7.8 ± 0.9 %, respectively, from the densitometric traces of protein (Fig. 2, solid line). As shown in Fig. 1B and Fig. 2 (broken line), at least eight different bands were recognized by the staining for the NADPH-diaphorase activity at the identical positions of protein

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                    5                   10                  15
Val-Glu-Lys-His-Ser-Arg-Lys-Met-Glu-Glu-Gly-Ile-Thr-Val-Asn-
                   20                  25
Lys-Phe-Lys-Lys(Thr)Lys-Lys-Tyr-Val-Gly-
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Fig. 3 N-terminal amino acid sequence of the main component (FNR-V) of ferredoxin-$NADP^+$ reductase.

staining. This implies that all of the protein bands have the enzyme activity as described by Gozzer et al. (5).

After extraction from the gel, all the component enzymes could be adsorbed strongly on the ferredoxin-Sepharose 4B affinity column, indicating a tight reductase-ferredoxin complex formation, and had the characteristic absorption spectra of flavoprotein identical with that of the 'non-separated' enzyme. These results mean that all the isolated component enzymes have properties typical of a ferredoxin-$NADP^+$ reductase.

The N-terminal amino acid sequence of the main component enzyme (FNR-V) extracted from the electrofocused gels was determined by the automatic Edman degradation method (Fig. 3). It is to be noted that FNR-V had 9 basic amino acid residues (7 Lys, His, and Arg) and 3 acidic amino acid residues (3 Glu) out of 25 residues in the N-terminal region. No Pro, Cys, or Trp was present in the N-terminal region.

As the N-terminal residues, the 'non-separated' enzyme had Val, Lys, Ala, Asn, and His, of which Val was of the largest amount. FNR-V was found to have only Val as the N-terminal residue. However, FNR-III and FNR-IV each was found to have several amino acids as the N-terminal residues: Asp, Val, His, and Ser for FNR-III; His, Ser, Val, and Ala for FNR-IV. These results suggest that both FNR-III and FNR-IV may still contain microheterogeneous components.

In order to get information as to the microheterogeneity of the reductase, FNR-III stored under various conditions was subjected to isoelectric focusing and SDS-polyacrylamide slab gel electrophoreses. As shown in Fig. 4A, protein bands at the position of FNR-VII showing the NADPH-diaphorase activity were recognized with all samples. As seen in Table I, the component protein had already been present before the addition of a protease inhibitor (DFP). No significant changes were detected in the electrofocused patterns of the samples stored for 3 days at

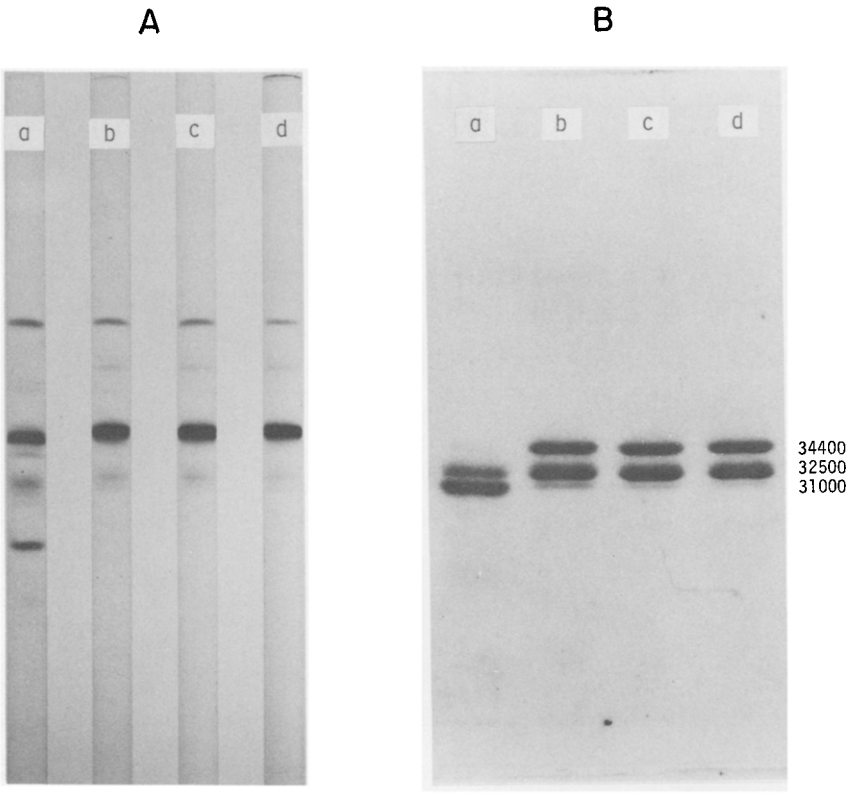

Fig. 4 (A) Isoelectric focusing of the component enzyme (FNR-III) after 31-day storage. The conditions were the same as in Fig. 1A. (B) SDS-polyacrylamide slab gel electrophoresis of the same samples as in A. The gel contained 10 % acrylamide and 0.1 % SDS, and was stained for protein. FNR-III (20 μM) was stored in 20 mM Tris-HCl buffer (pH 7.4) in the absence and presence of 1 mM DFP under the following conditions. a, 4 °C without DFP; b, 4 °C with DFP; c, -20 °C without DFP; d, -20 °C with DFP. The amounts of the reductase applied to the gels were 34 μg (A) and 1.1 μg (B), respectively.

4 °C (Table I). However, a new protein band at the position of FNR-I appeared only with the sample stored for 31 days at 4 °C in the absence of DFP (Fig. 4A, gel a). The sum of the amounts of components at the positions of FNR-I and FNR-III was almost identical with that of FNR-III of the samples stored under other conditions (Table I). From data in Fig. 4B, FNR-III was found to contain at least two components of molecular weights of 32,500 (51 %) and 34,400 (46 %). Since the component of the molecular weight of 34,400 was observed only with the isolated FNR-III and the amount of the component at the position of FNR-VII was about 20 % (Table I), the newly isolated FNR-III is considered to contain the different components having the different molecular weights but with the identical or very

Table I Relative Amounts of Protein Bands Recognized on Isoelectric Focusing Gels with the Component Enzyme (FNR-III) Stored under Various Conditions.

Time (day)	Temperature (°C)	Addition of DFP	Relative amount (%)		
			I	III	VII
0	4	-	0	75.3	24.7
0	4	+	0	78.8	21.2
3	4	-	0	79.1	20.9
3	4	+	0	79.3	20.7
31	4	-	31.0	49.6	19.4
31	4	+	0	81.2	18.8
31	-20	-	0	82.0	18.0
31	-20	+	0	85.4	14.6

Values were calculated from the densitometric traces of protein. I, III, and VII mean the protein bands at the positions of FNR-I, III, and VII.

close isoelectric points. Only with the sample stored for 31 days at 4 °C in the absence of DFP, the component of the greater molecular weight disappeared and a new protein band of a molecular weight of 31,000 appeared (Fig. 4B). These results indicate that a highly purified enzyme sample is still contaminated with trace amounts of a protease. Since Gozzer et al. (5) have reported that the different molecular species of the reductase were clearly identifiable after the first step of the purification procedure, it is conceivable that the protease was endogenous rather than exogenous, and could not be eliminated by the present purification methods. These lines of consideration lead to the conclusion that one reason for the molecular heterogeneity of the reductase is the digestion with the protease present in spinach leaves.

The isolated FNR-IV contained the main component of a molecular weight of 32,500 and a microheterogeneous component of a molecular weight of 31,000. The newly isolated FNR-V used for the N-terminal sequence analysis had a molecular weight of 32,500 and was the most homogeneous judging from the isoelectric focusing and SDS electrophoretic patterns. However, the isolated FNR-V was also converted more slowly into other forms than the other components. That the converted components were not focused on one component makes the interpretation of the molecular heterogeneity of the reductase quite complicated.

It has been established that ferredoxin-$NADP^+$ reductase tightly binds to ferredoxin under low ionic strength conditions but not under high ionic strength conditions (9, 17). Many basic amino acid residues were found in the N-terminal region of the reductase; especially, a cluster of basic amino acid residues was present in the range of Lys-16 to Lys-22 (Fig. 3). Since ferredoxin is quite an acidic protein (18), this range is a possible candidate for the ferredoxin binding site.

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