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SEPARATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE FERREDOXIN–THIOREDOXIN SYSTEM PROTEINS

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SUMMARY

In order to separate the four proteins of the ferredoxin–thioredoxin system, their behaviour was studied on different high-performance liquid chromatographic columns. When the proteins were not activated, the mixture could be totally resolved by gel filtration chromatography on a TSK 3000 SW column, using a phosphate buffer containing 0.3 *M* sodium chloride, or by anion-exchange chromatography. Hydrophobic interaction chromatography did not allow a one-step separation. When the proteins were light activated and their cysteinyl residues derivatized with iodoacetate or iodoacetamide, filtration on the TSK 3000 SW column was found to be the only efficient method for separating the four proteins in a one-step process.

INTRODUCTION

Several plant chloroplast enzymes require activation by light in order to be catalytically active[1,2]. One mechanism responsible for the light regulation consists in a cascade of thiol-disulphide interchanges through the so-called ferredoxin–thioredoxin system. This system is composed of three different proteins: ferredoxin (Fd), ferredoxin–thioredoxin reductase (FTR) and thioredoxin (TRX)[3]. Among the activated target enzymes (fructose-1,6-bisphosphatase, phosphoribulokinase, sedoheptulose-1,7-bisphosphatase, etc.) we have more closely studied the corn NADP-dependent malate dehydrogenase (NADP–MDH). In our laboratory, all the proteins involved in the light regulatory process are routinely purified, and a chloroplastic reconstituted system functioning *in vitro* has been developed, including photosynthetic membranes, Fd, FTR, TRX and NADP–MDH[4].

It has been shown that, during the light modulation process, NADP–MDH undergoes a post-translational modification, consisting in the reduction of one disulphide bridge on each subunit of the enzyme[5,6]. However, very little information is available about the intramolecular modification of the other proteins of the ferredoxin–thioredoxin system in the light-activated state, or about the redox regulation of this system. In order to obtain this information, it is necessary to isolate each of the

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four proteins of the system after reconstitution, light activation and thiol group derivatization by iodoacetic acid or iodoacetamide. Preliminary experiments have shown that NADP–MDH was unstable under these conditions, which excluded traditional purification procedures (1 week). Moreover, these procedures did not allow good yields with analytical injections. Hence a rapid and nearly quantitative procedure was necessary, which led us to develop a high-performance liquid chromatographic (HPLC) method. For this purpose we tested the separability of the four proteins of the ferredoxin–thioredoxin system by hydrophobic interaction, gel filtration and ion-exchange chromatography. In this paper, we report the results obtained in each instance and describe a method for separating the four proteins in one step.

EXPERIMENTAL

Proteins

Corn NADP–MDH was purified as described previously[7] and ferredoxin, ferredoxin–thioredoxin reductase and thioredoxin *m* were purified from spinach leaves as described[8–10]. Pea thylakoids were purified and stabilized as reported earlier[11].

HPLC

HPLC was performed at 20°C on a Laboratory Data Control system equipped with two minimetric pumps and a variable-wavelength UV detector monitored by a microcomputer (CCM). The following columns were used: TSK Phenyl 5PW, 75 × 7.5 mm I.D., (LKB) for hydrophobic interaction HPLC; TSK 2000 SW and TSK 3000 SW, 300 × 7.5 mm I.D., (Beckman) for gel-filtration (size-exclusion) HPLC; and poly(N-vinyldiimidazole) (PVDI), 100 × 4.6 mm I.D., (Société Française de Chromato Colonne) for anion-exchange HPLC. Proteins were detected at 280 nm.

Light-activation medium

The incubation medium (150 μl final volume) contained Tris–HCl, pH 8 (15 μmol), ferredoxin (23 μg), FTR (15 μg), thioredoxin (31 μg), NADP–MDH (32 μg) and chloroplast thylakoids (equivalent to 50 μg of chlorophyll). The activation was carried out for 15 min under nitrogen at 25°C under constant illumination at 300 W/m^2. Derivatization of new thiols was performed with [^{14}C]iodoacetic acid or [^{14}C]iodoacetamide for 15 min in the dark. Thylakoid membranes were removed by centrifugation and the supernatant injected directly on to the HPLC column.

Enzyme activity determination

After chromatographic separation, NADP–MDH was identified by its activity after activation in the presence of pure thioredoxin reduced by DTT[7]. TRX was identified by its ability to activate NADP–MDH in the presence of DTT[10]. FTR activity was determined in a complete reconstituted light-activation system[5] and Fd activity by measuring cytochrome *c* reduction in the presence of NADPH and pure ferredoxin–NADP reductase[12].

RESULTS AND DISCUSSION

Non-activated proteins

In a preliminary step, we studied the separation of the four chloroplastic pro-

TABLE I

RETENTION TIMES OF THE FOUR CHLOROPLASTIC PROTEINS AT TWO DIFFERENT FLOW-RATES ON A TSK PHENYL 5PW COLUMN

A linear 20-min gradient was used.

Flow-rate (ml/min)	*Retention time (min)*			
	NADP–MDH	*TRX*	*FTR*	*Fd*
1	20.0	20.0	4.2	9.4
2	17.2	17.8	4.2	6.3

teins without activation or subsequent derivatization. Several HPLC column supports were tested.

Hydrophobic interaction. A TSK Phenyl 5 PW column was used, and elution was performed with decreasing ammonium sulphate concentration from 1.8 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.2) using a linear gradient.

In Table I are reported the retention times (t_R) of the four proteins (Fd, FTR, TRX and NADP–MDH). Two flow-rates were tested with a 20-min gradient time. In this chromatographic system, FTR and Fd were well resolved, whereas NADP–MDH and TRX had similar retention times. As shown in Table I, increasing the flow-rate did not improve the separation significantly.

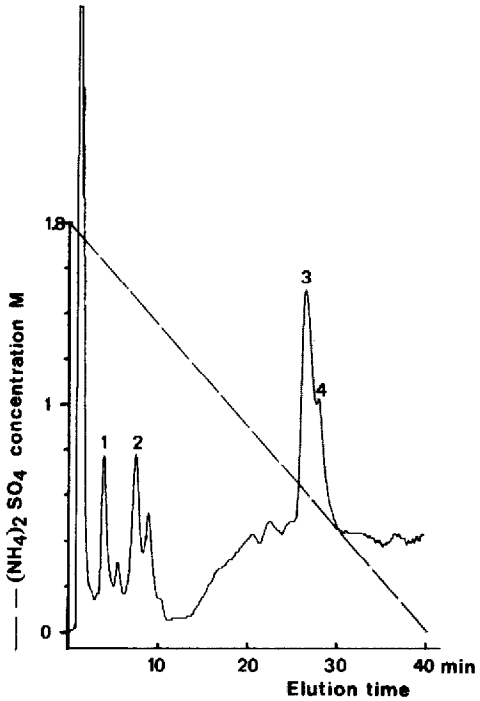

Fig. 1. Chromatogram of a protein mixture containing FTR (peak 1), Fd (peak 2), NADP-MDH (peak 3) and TRX (peak 4), obtained on a TSK Phenyl 5PW column at a flow-rate of 2 ml/min with a 40 min-linear gradient.

Variation of t_R with the gradient time, from 20 to 90 min, was investigated: TRX and NADP–MDH always coeluted (data not shown). Fig. 1 shows a typical chromatogram of the four-protein mixture, obtained with a 40-min gradient time and a flow-rate of 2 ml/min. As checked by absorbance measurement at 280 nm, the recovery of each protein ranged from 80 to 90%.

Gel filtration. As the molecular masses of NADP–MDH and TRX are different (80 and 12 kDa, respectively), the mixture of these two proteins should be resolved by gel filtration. We therefore tested two filtration columns, TSK 2000 SW and TSK 3000 SW.

Fig. 2 shows the separation of NADP–MDH from TRX on a TSK 2000 SW column, eluted with 30 m*M* Tris–HCl buffer (pH 7.5) at a flow-rate of 0.5 ml/min. The two proteins eluted as sharp peaks; NADP–MDH eluted first at 15 min, followed by TRX, which was retained for 19 min. On this matrix, NADP–MDH and FTR (30 kDa) were not completely separated (data not shown).

Hence hydrophobic interaction chromatography followed by gel filtration on TSK 2000 SW allows the complete resolution of the four-protein mixture. If gel filtration columns are not available, separation of NADP–MDH from TRX can also be achieved rapidly be several ultrafiltrations on a Centricon 30 microconcentrator, which selectively retains proteins having a molecular mass >30 kDa. NADP–MDH is recovered in the retentate in a concentrated form and TRX is recovered in the filtrate and can be concentrated on a Centricon 10 microconcentrator.

Table II shows the molecular masses of the four proteins and their corresponding retention times when each protein was injected separately onto a TSK 3000 SW

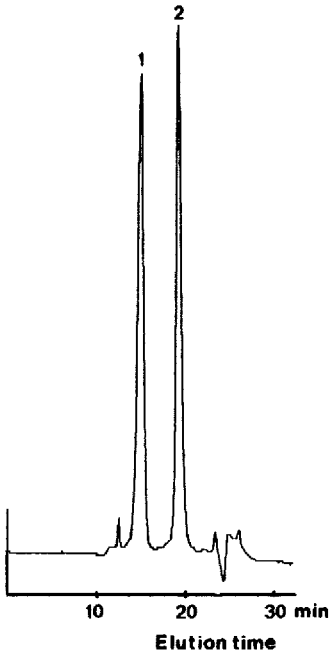

Fig. 2. Chromatogram of a protein mixture containing NADP–MDH (peak 1) and TRX (peak 2), obtained on a TSK 2000 SW column at a flow-rate of 0.5 ml/min.

TABLE II

MOLECULAR MASSES (MM) AND RETENTION TIMES OF THE FOUR CHLOROPLASTIC PROTEINS AFTER INJECTION OF EACH PROTEIN SEPARATELY ON A TSK SW 3000 COLUMN

The column was equilibrated with 30 m*M* Tris–HCl (pH 7.9) and the elution was performed with the same buffer at a flow-rate of 0.5 ml/min.

Protein	*MM (kDa)*	t_R *(min)*
NADP–MDH	80	12.6
Fd	11	13.8
FTR	32–34	14.7
TRX	12	16.9

column. Fd and TRX, having the same molecular mass, were expected to coelute on this column. However, Fd presents an abnormal retention time, as it eluted before FTR and TRX, hence with an apparently higher molecular mass than expected. With respect to this phenomenon, it is interesting that in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Fd migrates atypically as a 20-KDa protein[13].

Although the four proteins exhibited different retention times when injected individually, the elution profile obtained when the mixture was injected showed only three peaks (Fig. 3A), which means that two proteins coeluted. In order to determine which proteins coeluted, different mixtures of three proteins were tested: NADP–MDH + Fd + TRX (Fig. 3B) and NADP–MDH + FTR + TRX (Fig. 3C). In both instances three peaks were obtained, with the expected retention times. However, NADP–MDH + FTR + Fd (with Fd in excess over FTR) showed only two peaks with t_R = 12.6 and 13.7 min, corresponding to NADP-MDH and Fd, respectively (data not shown). On the other hand, the mixture of Fd and FTR with a 1:1 stoichiometry showed only one peak with a shorter t_R (12.4 min) (data not shown). This set of results strongly suggests that Fd and FTR form a complex that is eluted with the same t_R as NADP–MDH. This is not surprising as FTR is routinely purified by affinity chromatography on a ferredoxin – Sepharose column[9]. This hypothesis was confirmed by performing the same experiment in the presence of 0.3 *M* sodium chloride in order to dissociate the complex: two peaks were observed, which corresponded to FTR (t_R = 19.7 min) and Fd (t_R = 21 min), these values being identical with those observed with separate injections of each protein in the presence of sodium chloride (data not shown). Remarkably, under these conditions Fd was retained for a longer time than FTR, in contrast with its behaviour in the absence of sodium chloride.

These results are in agreement with those recently reported by Hirasawa *et al.*[14], who showed that FTR formed an electrostatically stabilized 1:1 complex with Fd.

Following these observations, the mixture of the four proteins was then chromatographed on a TSK 3000 SW column in the presence of 0.3 *M* sodium chloride. Four peaks were obtained (Fig. 4); each was tested for its enzymatic activity and its purity checked by SDS-PAGE. The results are summarized in Table III. As checked by absorbance measurement at 280 nm, the recovery of each protein was about 90%.

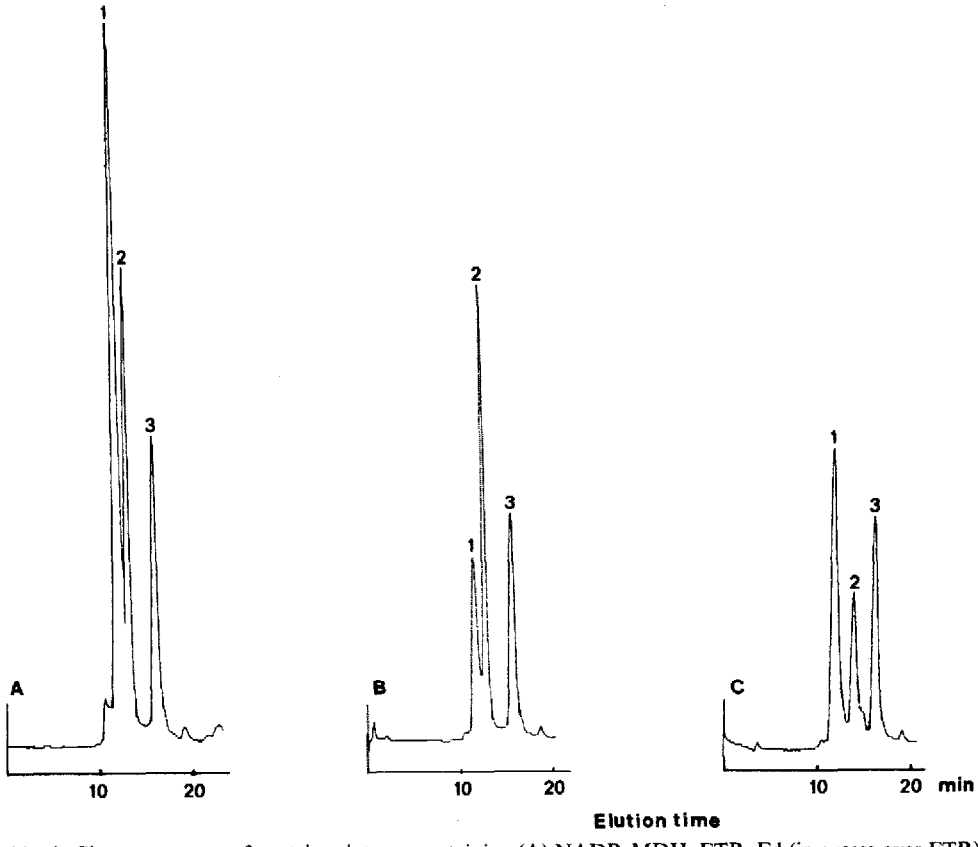

Fig. 3. Chromatograms of protein mixtures containing (A) NADP–MDH, FTR, Fd (in excess over FTR) and TRX, (B) NADP–MDH (peak 1), Fd (peak 2) and TRX (peak 3) and (C) NADP–MDH, (peak 1), FTR (peak 2) and TRX (peak 3), obtained on a TSK 3000 SW column at a flow-rate of 0.5 ml/min.

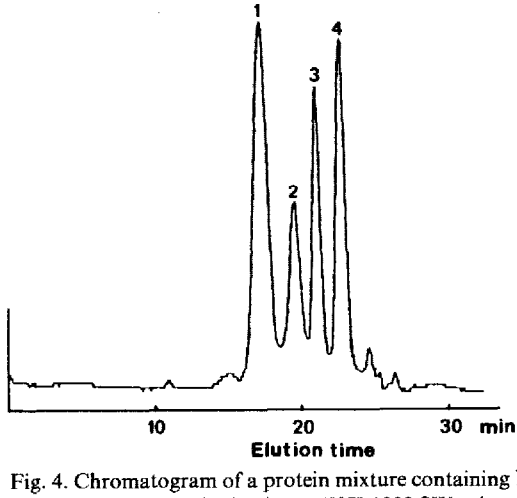

Fig. 4. Chromatogram of a protein mixture containing NADP–MDH (peak 1), FTR (peak 2), Fd (peak 3) and TRX (peak 4), obtained on a TSK 3000 SW column at a flow-rate of 0.5 ml/min in the presence of 0.3 *M* sodium chloride.

TABLE III

RETENTION TIMES, ENZYMATIC ACTIVITIES AND ELECTROPHORETIC ASSIGNMENTS OF THE FOUR PEAKS OBTAINED AFTER CHROMATOGRAPHY OF THE PROTEIN MIXTURE ON A TSK 3000 SW COLUMN IN THE PRESENCE OF 0.3 *M* SODIUM CHLORIDE

The column was equilibrated with 30 m*M* Tris–HCl (pH 7.9) containing 0.3 *M* sodium chloride and elution was performed with the same buffer at a flow-rate of 0.5 ml/min. The enzymatic activities were checked as described under Experimental.

Peak	t_R *(min)*	*Enzymatic activity*	*Electrophoretic assignment*
1	17.4	NADP–MDH	NADP–MDH
2	19.6	FTR	FTR
3	21	Fd	Fd
4	22.6	TRX	TRX

Hence, chromatography on TSK 3000 SW in the presence of 0.3 *M* sodium chloride allowed the rapid separation of the four proteins in only one step.

Ion exchange. The third matrix tested was an anion-exchange support, PVDI. Fig. 5 shows an elution pattern of the four-protein mixture obtained with a 30-min

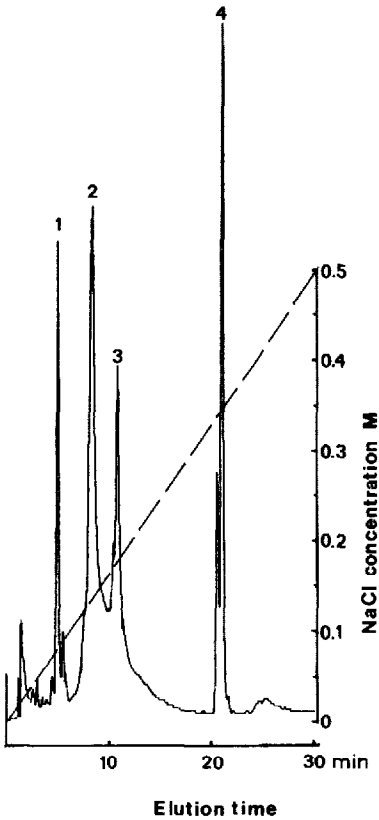

Fig. 5. Chromatogram of a protein mixture containing TRX (peak 1), NADP–MDH (peak 2), FTR (peak 3) and Fd (peak 4), obtained on a PVDI column at a flow-rate of 1 ml/min using a 30-min linear 0–0.5 *M* sodium chloride gradient in 30 m*M* Tris–HCl buffer (pH 7.5).

linear gradient from 0 to 0.5 *M* sodium chloride in Tris–HCl buffer. TRX eluted first, as a sharp peak, followed by NADP–MDH and FTR, and finally Fd at the end of the gradient. A minor contaminant of this Fd preparation, which eluted slightly before the major peak, was separated.

The variation of the t_R with the gradient time (from 20 to 60 min) was investigated. In this range, the four proteins were well resolved (data not shown). Therefore, a 30-min gradient can be used routinely to separate these four proteins and the recovery of each was *ca.* 70–80%. Similar results were obtained with a Mono-Q column (Pharmacia).

Hence a one-step separation was also achieved by ion-exchange chromatography on a PVDI column.

Light-activated proteins

In a second step, we studied the separation of the same proteins after light activation and thiol group derivatization with [^{14}C]iodoacetate. After such a treatment, FTR, TRX and NADP–MDH were recovered as ^{14}C-carboxymethylated proteins whereas Fd, which does not contain any derivatizable thiol group, was not modified[5]. The same columns were tested.

Hydrophobic interaction. Identical results were obtained as for non-derivatized proteins (data not shown).

Gel filtration. As the molecular masses are not significantly modified after derivatization, the same chromatographic behaviour was expected as for non-activated proteins. In fact, after activation and thiol derivatization performed with [^{14}C]iodoacetamide, the mixture was totally resolved on TSK 3000 SW in the presence of 0.3 *M* sodium chloride, although the peak containing derivatized NADP–MDH (peak 1) was slightly broadened (Fig. 6). SDS-PAGE revealed (data not shown) that each fraction had the same assignment when the proteins were derivatized, *i.e.*, NADP–MDH eluted first, followed by FTR (peak 2), Fd (peak 3) and TRX (peak 4). Derivatization with iodoacetamide did not lead to significantly different t_R in comparison with non-derivatized proteins. However, when derivatization was performed with iodoacetate, TRX showed a shorter t_R of 21.8 min instead of 22.6 min (data not shown). This unexpected behaviour could be explained by ionic interactions between the matrix of the column and the protein.

Ion exchange. Fig. 7 shows the elution profile obtained after injection of the mixture of light-activated proteins on a PVDI column using a 40-min linear gradient. Derivatization was performed with [^{14}C]iodoacetate, and each fraction was analysed by SDS-PAGE and its radioactivity counted. [1-^{14}C]carboxymethyl-TRX was recovered in a purified form in peak 1. Peak 2 (t_R = 14.3 min) was not radioactive and SDS-PAGE revealed that it contained NADP–MDH (i.e., the fraction of protein that had not been activated and hence had no thiol group available for derivatization). Peak 3 contained a mixture of [1-^{14}C]carboxymethyl-NADP–MDH and [1-^{14}C]carboxymethyl-FTR and, as expected, Fd was recovered in peak 4. In this assay, NADP–MDH was not totally reduced. In the cases where it was, peak 2 was not present in the elution pattern. The different retention time of carboxymethylated NADP–MDH, compared with underivatized NADP–MDH, could be explained by the presence of several new negative charges. Nevertheless, when iodoacetamide was used instead of iodoacetate, similar results were unexpectedly obtained.

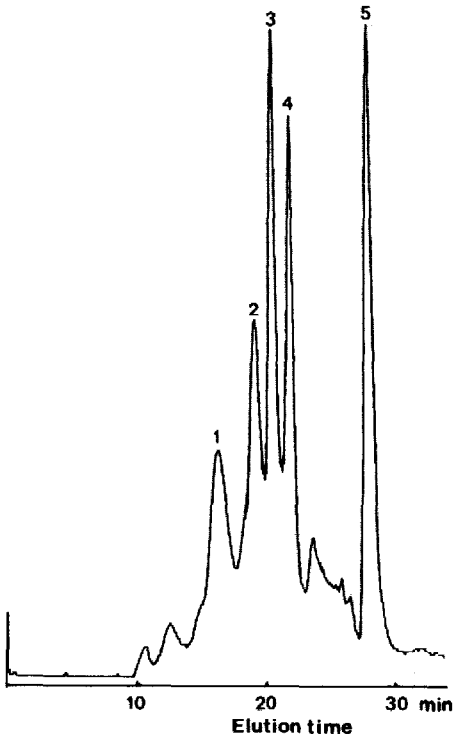

Fig. 6. Chromatogram of the light-activated protein mixture after derivatization with iodoacetamide, obtained on a TSK 3000 SW column at a flow-rate of 0.5 ml/min in the presence of 0.3 *M* sodium chloride. Peak 5: excess iodoacetamide.

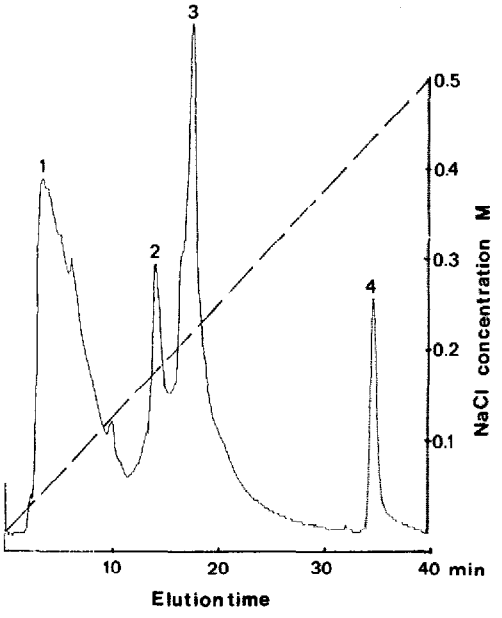

Fig. 7. Chromatogram of the light-activated protein mixture after derivatization with iodoacetate, obtained on a PVDI column at a flow-rate of 1 ml/min using a 40-min linear gradient.

Therefore, ion-exchange chromatography is less efficient when the protein mixture is activated and derivatized, as FTR and NADP–MDH cannot be separated.

CONCLUSION

Several HPLC column matrices (gel filtration, ion-exchange, hydrophobic interaction) have been tested in order to separate rapidly the four proteins involved in the *in vitro* enzyme light-activation system: thioredoxin, ferredoxin, ferredoxin–thioredoxinreductase and NADP–malate dehydrogenase. When proteins were not activated, hydrophobic interaction on a TSK Phenyl 5PW column allowed a good separation between FTR, Fd and a mixture containing TRX and NADP–MDH to be obtained. TRX was further resolved from NADP–MDH by gel filtration on a TSK 2000 SW column or by ultrafiltration on a Centricon 30 microconcentrator. Alternatively, the four-protein mixture could be completely resolved on a TSK 3000 SW column using a buffer containing 0.3 *M* sodium chloride.

Another attractive chromatographic system is anion-exchange chromatography on a PVDI or Mono-Q column, which allowed a one-step separation of each protein.

When proteins were light activated and then carboxymethylated, ion-exchange chromatography could not be used satisfactorily. However, gel filtration remained a rapid and efficient method for resolving the mixture, provided that sodium chloride is present in the filtration medium in order to dissociate the Fd–FTR complex.

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