Biochemistry and Molecular Biology Education
Vol. 31, No. 2, pp. 119–122, 2003

Laboratory Exercises

Purification of Colored Photosynthetic Proteins for Understanding Protein Isolation Principles

Received for publication, October 11, 2002, and in revised form, December 11, 2002

M. Teresa Bes, Javier Sancho, M. Luisa Peleato, Milagros Medina, Carlos Gómez-Moreno, and María F. Fillat‡

From the Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, 50009 Zaragoza, Spain

The purification of a protein is the essential initial step in the study of its physical and biological properties and is one of the most common procedures in biochemistry. This article describes a method for teaching purification skills through the partial isolation of ferredoxin-NADP$^+$ reductase and ferredoxin from a single cell batch. The method has been used for several years in an introductory biochemistry course using spinach leaves as cellular source. The protocol gives a complete picture of the preparation of a crude extract and the subsequent isolation of both electron transport proteins on a laboratory scale. It introduces students to the use of different techniques for the purification and detection of proteins and allows them to develop a number of valuable experimental and analytical skills without necessarily resorting to complicated or expensive equipment.

Keywords: Protein purification, photosynthetic proteins, chromatography, protein activity.

Laboratory experience helps students to develop their critical thinking and creativity. Moreover, by doing practical work they increase their appreciation of the mechanisms by which scientists obtain and analyze information. It is actually through the laboratory training that the students become intensely and personally involved in the acquisition of knowledge. Based on this idea, we have developed in the last years a short practical training on protein purification as part of a general biochemistry course.

Biochemical work frequently requires the purification of a particular compound from a complex mixture. In fact, proteins constitute one of the probable candidates to be handled or isolated by the biochemistry student along its professional carrier. As a model for developing this skill we have focused our interest on the purification of ferredoxin-NADP$^+$ reductase (FNR)[1] from spinach, which in addition allows the obtainment of the protein ferredoxin as a side product. Both proteins are colored (FNR is yellow, and ferredoxin is brown), relatively abundant in the starting material, easy to handle, and present pronounced biochemical differences, which enable students to make interesting comparisons.

FNR is the FAD-containing enzyme responsible for NADP$^+$ photoreduction in plants, green algae, and cyanobacteria [1]. It has been isolated from many sources and extensively characterized. The molecular mass of FNR from spinach is 36,000 Da, and its isoelectric point is around 5. The ability of FNR to reduce artificial substrates such as 2,6-dichlorophenolindophenol (DCPIP) or ferricyanide (diaphorase activity) using NADH or NADPH as electron source [2] allows easy recognition of this enzyme in any stage of purification.

Ferredoxin is a small iron-sulfur electron carrier protein that performs the transfer of electrons from photosystem I to FNR and is involved in many other biological electron transport chains in cyanobacteria and plants and is widely distributed in bacteria also [3].

Because of the special characteristics of both proteins and their simultaneous presence in the spinach leaves, a starting material that is cheap and readily available all over the world, we consider this is an excellent system for teaching the principles of protein purification. In the practice, the student is instructed in the logical process of purification, which starts from a raw material by applying some preparatory steps of purification. Later the purification is refined, and the purity of the product obtained is evaluated. At the same time, students are trained in some important biochemical aspects such as (*a*) the study of the spectral properties of a protein, (*b*) the determination of the time course of product formation in an enzyme-catalyzed assay with calculation of activity units, (*c*) the measurement of protein concentration based on values obtained from indirect data using different dilutions, (*d*) the determination of a protein molecular weight by SDS-polyacrylamide gel electrophoresis, or (*e*) the elaboration of a written report.

This practice has been designed to be performed in a short time, and the protein preparation obtained is only

‡ To whom correspondence should be addressed. Tel.: 34-976-761282; Fax: 34-976-762123; E-mail: fillat@posta.unizar.es.

[1] The abbreviations used are: FNR, ferredoxin-NADP$^+$ reductase; DCPIP, 2,6-dichlorophenolindophenol.

partially purified. However, we explain that in preparative separations, the main aim is to isolate and recover an amount as large as possible of the compound in a high degree of purity to subsequently study its chemistry and/or its biological properties. Students also learn that any purification procedure adopted will inevitably involve some loss of material and that it is essential that the number of purification steps should be kept to a minimum, and therefore the techniques used should be those that are capable of giving the greatest purification yield.

To provide the students a basic background for a better understanding of this practice, it is preceded by a short theoretical training on the principles of protein purification. Moreover, students are encouraged to read and learn more about protein purification methods and analysis in the biochemistry lab books available at the university. We mainly use the texts of Wilson and Walker [4] and Boyer [5]. The development of this practical work has shown to be very useful to pose questions whose finality is to encourage students to think about what is observed and to stimulate the imagination of the student. We have verified that this constitutes a good method for a didactic teaching of sciences.

ORGANIZATION

This practical can be completely performed by the students working in pairs in five sessions of ~3–4 h each, or six sessions if electrophoresis is included, using consecutive days. In the first session the students prepare the crude extract and fractionate it with acetone. It is stored in the refrigerator to be used the following day. In the second session a hand-made DEAE-cellulose ionic exchange chromatography column is prepared as well as a Cibacron blue Sepharose affinity column. In the third session both chromatographies are performed. In sessions four and five a purification table is elaborated based on the data obtained from the different samples stored in the freezer after every purification step. For this purpose, the students determine the protein content of the samples using the method of Lowry. They also measure the diaphorase activity of FNR, and they record the UV-visible spectra of the partially purified samples. The work can be completed in a sixth session by performing a 12% SDS-polyacrylamide gel electrophoresis from the aliquots obtained in the purification process to visualize the progression of the purification and, if desired, to introduce students in the determination of protein molecular weights. Finally, the students discuss their results and elaborate a short written report.

EXPERIMENTAL PROCEDURES

Equipment Required—The practice can be carried out with the equipment and supplies commonly found in biochemistry teaching laboratories. The major pieces of equipment are a spectrophotometer with UV lamp, preferably with an attached recorder for timed reactions, and a centrifuge. Chromatography work can be carried out with home-made glass or plastic tubes or with a syringe using manual fraction collecting. Routine laboratory equipment such as a balance, pH meter, stirrer, and freezer are also required. Volumetric measurements can be made with graduated cylinders and pipettes. For small amounts, the use of adjustable Eppendorf-type pipettes with disposable tips is desirable.

Crude Extract Preparation—The general scheme of purification followed along this practice is shown in Fig. 1. 75 g of spinach leaves are mixed with 110 ml of 50 mM Tris/HCl buffer, pH 8 and triturated using a Waring blender for 2 min. The resulting homogenate, which from this moment must be kept in an ice bath, is filtered through a double cheese cloth placed in a funnel to eliminate the non-soluble debris. The green solution obtained is considered the crude extract. The volume of this extract is measured before removing 3 ml (aliquot 1) that will be kept in the freezer for subsequent analysis.

Acetone Fractionation—The crude extract is mixed, using gentle stirring, with a volume of acetone (precooled to −15 °C) to give a final concentration of 35% (v/v). Added volume = 35 × crude extract volume/(100 − 35).

The mixture is centrifuged at 4 °C for 10 min at 5,000 rpm. After centrifugation the precipitate is discarded, and a volume of precooled acetone is added to the supernatant to give a final concentration of 75% (v/v). Added volume = [(75 − 35)/(100 −35)] × Present volume (the student has to measure it). This mixture is kept in the refrigerator to be used the following day.

Crude Extract Dialysis—Most of the acetone is removed from the above "settled suspension" by suction using a pump. The remaining suspension is centrifuged at 4 °C for 10 min at 5,000 rpm. The supernatant is removed, and the pellet is allowed to dry at room temperature in the hood for a couple of hours or until the acetone smelt has vanished. A homogenate is prepared by suspending the obtained acetone powder in the smallest volume of 5 mM Tris/HCl buffer, pH 8 (around 10–20 ml). This homogenate is dialyzed against 5 liters of the same buffer, which are renewed 12 h later.

Chromatography Column Preparation—Although many commercial columns are available, students use simple and inexpensive materials for preparing chromatography columns in the laboratory. In the case of the DEAE-cellulose column, they use a glass or plastic tube (12 × 1.5 cm) whose ends are sealed with rubber stoppers. The stoppers are previously drilled to introduce a plastic tip in the hole. A filter paper disc is used to seal the stopper and acts as the base of the column before it is vertically placed in a holder. Rubber tubing connected to the tip and provided with a clamp allows the student to regulate the column flow. A suspension (1:3) of DEAE-cellulose (Whatman DE-52) in 50 mM Tris buffer, pH 8 is added to the column until the packed material reaches around 5 cm in height. The column is closed and connected to a reservoir containing 50 mM Tris buffer, pH 8 and washed with 5 column volumes.

The Cibacron blue Sepharose column is packed in a glass column (10 × 1.5 cm). As previously described, rubber tubing connected to the base and provided with a clamp will allow the student to regulate the column flow. The column is equilibrated with 5 volumes of 50 mM Tris/HCl buffer, pH 8.

Chromatography in DEAE-cellulose—The dialyzed crude extract is centrifuged at 4 °C for 10 min at 14,000 rpm to clarify the solution. The supernatant, containing FNR and ferredoxin, is separated from the pellet, and the volume of this extract is measured before removing 1 ml (aliquot 2) that will be kept in the freezer for subsequent analysis.

The suspension is poured into the equilibrated DEAE-cellulose column using a Pasteur pipette and washed with 15 ml of 50 mM Tris/HCl buffer, pH 8 or until all colored moving bands are removed from the column. FNR and ferredoxin are anionic proteins, and consequently they will remain fixed in the column after washing.

FNR is eluted from the column using 30 ml of 50 mM Tris/HCl buffer, pH 8 containing 0.15 M NaCl. During the elution, a yellow band containing FNR moves along the column, while a brownish band containing ferredoxin remains on the top of the column. 2-ml fractions containing FNR (recognized by its yellow color) are pooled, and the volume is measured. Then 0.3 ml (aliquot 3) are removed and frozen for subsequent analysis.

Elution of the ferredoxin-containing brownish band is per-

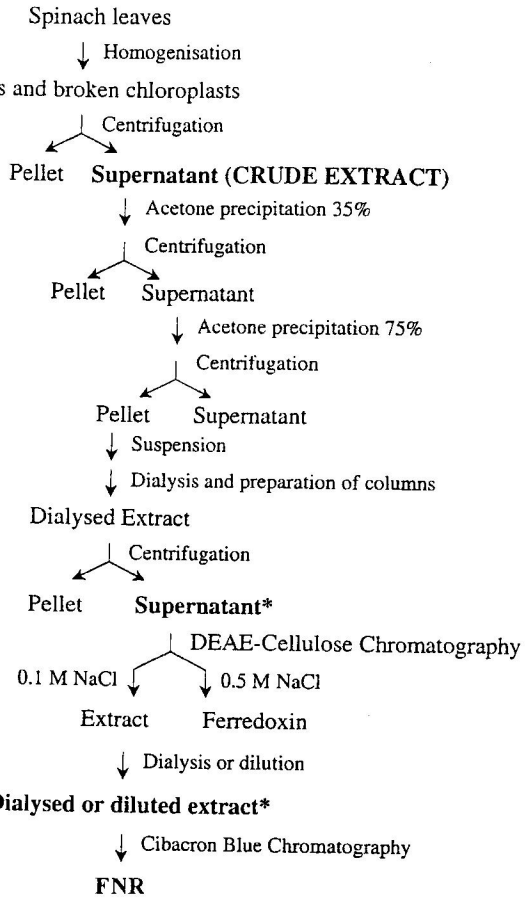

FIG. 1. **General scheme of the different steps followed in the course of the practical work.** *, to elaborate the purification table, 1 ml of solution is removed after measuring the total volume of the solution in these steps.

formed by washing with 50 mM Tris/HCl buffer, pH 8 containing 0.5 M NaCl. In this step, a partially purified ferredoxin sample is obtained.

In this chromatography and in the next one, the student's work can be improved if absorbances at 458 nm are recorded to select the tubes that contain FNR. This wavelength corresponds to the maximum of absorbance of spinach FNR in the visible region of the spectrum.

Chromatography in Cibacron Blue Sepharose—The pooled fractions containing FNR are diluted with 1 volume of 50 mM Tris/HCl buffer. This solution is poured into the previously prepared Cibacron blue Sepharose column [6]. After washing with 15 ml of 50 mM Tris/HCl buffer, pH 8, all the proteins not showing affinity to the matrix will be eluted, while FNR is retained. By increasing the ionic strength by washing with 30 ml of 50 mM Tris/HCl buffer, pH 8 containing 0.5 M NaCl, FNR is eluted, and 2-ml fractions are collected. The best three fractions are selected based on the intensity of the color and are mixed. The volume is measured, and the aliquot is frozen (aliquot 4). Aliquot 4 corresponds to the final purified FNR.

Protein Analysis—Quantitative protein determinations are performed by the Lowry procedure [7]. For the described purification protocol a standard calibration curve is calculated from six points corresponding to 0.5, 0.3, 0.2, 0.1, 0.05, and 0.0 mg/ml bovine albumin. The different aliquots are diluted to obtain absorbance values that fit inside the calibration curve.

FNR Diaphorase Activity Assay—FNR is quantified in the different samples by calculating its diaphorase activity, that is, measuring electron transfer from NADPH to DCPIP [2]. The diaphorase activity of an aliquot is obtained by determining the decrease in absorbance at 620 nm, considering $\epsilon_{DCPIP}^{620\ nm} = 21$ $mM^{-1}\ cm^{-1}$. In a typical assay the following amounts are used. In the reference cuvette, 800 μl of 50 mM Tris/HCl buffer, pH 8 and 200 μl of 0.19 mM DCPIP are used. In the sample cuvette, 390 μl of 50 mM Tris/HCl buffer, pH 8, 500 μl of 0.19 mM DCPIP, 100 μl of 0.5 mM NADPH, and 10 μl of sample are added and mixed immediately before starting the measurement. With the exception of the crude extract, a 1:4 dilution for all the aliquots is advisable. Measurements are performed at room temperature.

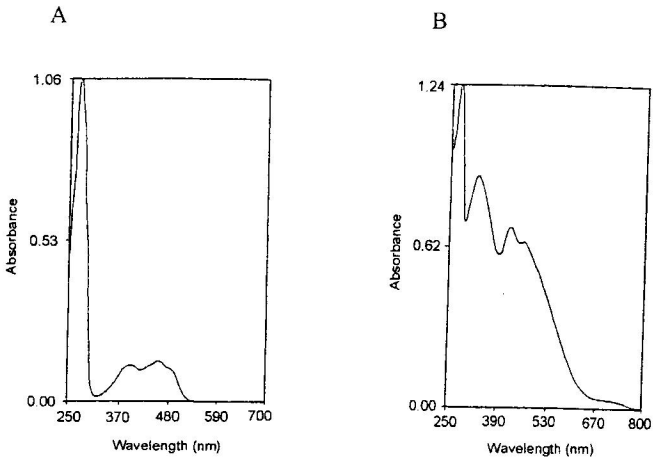

FIG. 2. **UV and visible spectrum of ferredoxin-NADP$^+$ reductase (*A*) and ferredoxin (*B*) from spinach leaves in 50 mM Tris/HCl buffer, pH 8.0.**

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis is performed according to Laemmli [8] using 12% (w/w) polyacrylamide (30:0.8 (w/w) acrylamide/bisacrylamide) gels. After electrophoresis the gel is stained with Coomassie R (0.025 g/liter in 45% methanol, 6% acetic acid).

RESULTS AND DISCUSSION

The laboratory practice proposed here has proved to be useful for undergraduate students to understand protein purification principles, although the obtained protein preparation is only partially purified. The advantages of using these proteins are that students deal with two colored proteins that allow them to follow their movement along the columns by direct observation, and, unlike ferredoxin, FNR is an enzyme, and its diaphorase activity is easily measured so that the students are able to elaborate a purification table. The volume of crude extract obtained by students is small, around 110 ml, and they have no difficulties in handling it.

Normally the purification of a protein involves the application of one or more chromatographic steps, each of which generates a relatively large number of test tubes (fractions) containing buffer and protein eluted from the column. In this practice, the size of the columns has been scaled to avoid large volumes and to recover the protein sample in no more than three test tubes. A method is also required to determine which tubes contain the protein of interest so that their content can be pooled and used for the next purification step. With the use of two colored proteins this is simplified since the students simply observe and recover the fractions. However, the instructor should point out that this approach (the eye substitutes for the spectrophotometer) can only be done because of the special characteristics of these proteins and that proteins normally lack color. Consequently, the use of the spectrophotometer is normally necessary at this stage.

TABLE I

Example of a FNR purification schedule obtained by students who have performed this practice using spinach leaves as cellular source

Fraction	Volume	Total protein	Total activity[a]	Specific activity	Purification factor[b]	Overall yield[c]
	ml	*mg*	*IU*	*IU mg^{-1}*		%
Crude extract	120.5	1488.2	139.9	0.094	1	100
Acetone fractionation	17.5	89.25	79.7	0.89	9.5	57
DEAE-cellulose	40	47.6	63.6	1.34	14.2	45
Cibacron blue sepharose	6	5.1	43.1	8.51	90.6	31

[a] The unit of enzyme activity (IU) is defined as the amount that produces 1 μmol of product/min under standard assay conditions.
[b] Purification factor = (specific activity of fraction/specific activity of crude extract).
[c] Overall yield = (total activity of fraction/total activity of crude extract).

In the first column, the differences in the behavior of two anionic proteins can be clearly distinguished, and the student gets the idea of how a protein elutes from a column by using a salt gradient. From this first column, the pool of FNR shows an intense yellow color because of the presence of the prosthetic group FAD, although comparison of its spectrum with that of the pure enzyme (Fig. 2*A*) gives the students the idea of other non-colored contaminants that are also present in their preparations.

It is after the second column that the students get a FNR preparation with a spectrum comparable to that of the pure enzyme that shows a purity ratio, $A_{274\ nm}/A_{458\ nm}$, around 11 (*versus* 8 for the pure enzyme [9]). This indicates to the students that the quality of the protein preparation has improved compared with the result from the first column, although it is not completely pure.

Ferredoxin obtained from the DEAE column after washing with 50 mM Tris/HCl buffer, pH 8 containing 0.5 M NaCl presents a brownish color typical of ferredoxin. Comparison of its spectrum with the one of pure ferredoxin (Fig. 2*B*) reveals that the protein is contaminated with some uncolored components, mostly nucleic acids, as revealed by the high absorbance of this sample at 250 nm [5]. The purity ratio, $A_{274\ nm}/A_{420\ nm}$, for pure ferredoxin from spinach is 2.04.

It is interesting to note that, using the purity ratio previously calculated for the pure species, the use of electrophoresis to analyze the purity of the samples can be avoided. This is possible because of the special spectral properties of both proteins. However, performance of the SDS-polyacrylamide gel electrophoresis is recommended because of the power of this technique to analyze complex protein mixtures and the possibility of determining the FNR molecular weight.

Once the experiment has been completed, students have four aliquots that they need to analyze to determine the total protein content and enzyme activity since they have to elaborate the purification table. Protein content is measured using the Lowry method so that students learn to calculate protein concentration using a calibration curve and practice the use of different dilutions from a sample. Nevertheless, other alternative methods for total protein quantification can be used.

The enzymatic activity of the fractions is calculated using a quite simple diaphorase activity assay that takes only 1 min so that long waiting times in front of the spectrophotometer are avoided. Moreover, this assay is based on color changes at 620 nm so that plastic cuvettes can be used, avoiding the use of the more expensive quartz cuvettes.

A typical purification table obtained by students after finalizing this practice is shown in Table I. From the table it would be clear to them how the purification process has gone. At the beginning they had around 120 ml of crude extract that have become only 6 ml of enriched enzyme solution as can be deduced by comparison with the total and specific activity columns. Although they lose quite a large amount of protein along the experiment, the result is good because in only three steps they purify their protein preparation 90-fold.

Along this practice, we have observed that the students appear to be very motivated, and frequently they compare their results with those obtained by other students. This allows them to think and become critical of their own work.

REFERENCES

[1] D. Voet, J. Voet, C. Pratt (1999) Fundamentals of Biochemistry, Wiley, New York, pp. 529–548.
[2] G. Zanetti, B. Curti in San Pietro, A., Ed. (1986) Methods in Enzymology, Academic Press, New York, Vol. 69, pp. 250–255.
[3] D. L. Nelson, M. M. Cox (2000) Lehninger: Principles of Biochemisty, 3rd ed., Worth Publishers, New York, pp. 746–761, 819–826.
[4] K. Wilson, J. Walker (2000) *Principles and Techniques of Practical* Biochemistry, 5th ed., Cambridge University Press, Cambridge.
[5] R. Boyer (2000) Modern Experimental Biochemistry, 3rd ed., Benjamin/ Cummings, San Francisco, pp. 29–140.
[6] J. Naval, M. Calvo, F. Lampreave, A. Piñeiro (1983) Affinity chromatography of serum albumin: an illustrative laboratory experiment on biomolecular interactions, *Biochem. Educ.* **11**, 5–8.
[7] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randal (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* **193**, 265–275.
[8] U. K. Laemmli (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680–685.
[9] J. Sancho, M. L. Peleato, C. Gómez-Moreno, D. E. Edmondson (1988) Purification and properties of ferrodoxin-NADP$^+$ oxidoreductase from the nitrogen-fixing cyanobacteria Anabaena variabilis, *Arch. Biochem. Biophys.* **260**, 200–207.