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Development of an ELISA approach for the determination of flavodoxin and ferredoxin as markers of iron deficiency in phytoplankton

Luis A. Inda, M. Luisa Peleato*

Departamento de Bioquímica, Facultad de Ciencias, Universidad de Zaragoza, 50009-Zaragoza, Spain

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Abstract

Quantification of the iron-nutritional status of phytoplankton is of great interest not only for the study of oceans but also for fresh waters. Flavodoxin is a small flavoprotein proposed as a marker for iron deficiency, since it is induced as a consequence of iron deprivation, replacing the iron–sulphur protein ferredoxin. Flavodoxin and ferredoxin have been frequently used as markers for determination of iron deficiency in phytoplankton. Using purified flavodoxin and ferredoxin from *Scenedesmus vacuolatus* and polyclonal antibodies against both proteins, individual ELISA tests have been developed. The assays have a linear response in the range of 30–600 ng/ml of protein.

Keywords: ELISA; Ferredoxin; Flavodoxin; Iron deficiency; Phytoplankton

1. Introduction

Iron availability and biolimitation by iron have been an important focus of discussion for oceanographers, and after IronEX II (Coale et al., 1996; Behrenfeld et al., 1996; Kirchman, 1996), it was definitively established that availability of iron limits rates of cell division as well as abundance and production in phytoplankton of the equatorial Pacific and likely in other "high nutrient, low chlorophyll regions" (Frost, 1996). The estimation of the iron-nutritional status of phytoplankton has been an issue of great interest, not only for oceans but also for fresh waters, where toxin production (Lukac and Aegerter, 1995) or undesirable blooms can be related to iron availability.

The need to develop a rapid and concise assay for the diagnosis of iron deficiency in phytoplankton has been clear for some time. The ideal test should be rapid, specific to iron deficiency and without requiring large manipulations of biomass. Several approaches have been used as indicators of iron deficiency in natural populations, based upon the concept that an organisms response to environmental conditions often involves the synthesis of molecules that are unique to that condition (Falkowski et al., 1992). By detecting alterations in the abundance of such proteins or ribonucleic acids, the cell may be used as a reporter of the environment. Immunochemical detection has been widely used to detect phytoplankton physiological status (e.g. nitrogen metabolism, nutrient deficiencies), species diversity, symbiosis and community ecology and many other aspects related to marine ecology (see reviews Ward, 1990; LaRoche et al., 1999). One of the most studied responses to iron limitation is the induction of flavodoxin expression. Under iron deficiency in cyanobacteria and many algae, flavodoxin (containing FMN as the redox centre) replaces the iron-containing ferredoxin in photosynthetic electron transport and other electron transfer processes (e.g. nitrite reductase, glutamate synthase). Flavodoxin has been proposed as a suitable marker for evaluating the iron-nutritional status of phytoplankton in high-nutrient low-chlorophyll oceanic regions (La Roche et al., 1996; Doucette et al., 1996). Doucette et al. (1996) proposed the relative abundance of flavodoxin, as a percentage of the combined pool of flavodoxin and ferredoxin as a diagnostic indicator of iron stress (Flavodoxin Index). The Flavodoxin Index corrects heterogeneity of flavodoxin induction among organ-

* Corresponding author. Tel.: +34-976-762479; fax: +34-976-762123.
E-mail address: mpeleato@posta.unizar.es (M.L. Peleato).

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isms, with the presence of constitutive flavodoxin in several cases (Entsch et al., 1983).

The plant-type ferredoxins are [2Fe-2S] proteins which function as one-electron carriers between the membrane-bound iron–sulphur centres in photosystem I (PSI) and the different ferredoxin-dependent enzymes, such as ferredoxin-$NADP^+$ reductase (FNR), nitrite reductase, sulphite reductase, glutamate synthase and thioredoxin reductase. Ferredoxin is also involved in some proposed schemes for electron transport in cyclic photophosphorylation. In eukaryotes, apoferredoxin is encoded by a nuclear gene and is synthesized in the cytosol as a longer precursor (Huisman et al., 1978). The coordination of iron and sulphur by apoferredoxin to yield the holoprotein occurs in the stroma of the chloroplast once it has been imported and the transit peptide cleaved (Li and Merchant, 1992). Light is able to modulate ferredoxin gene expression in both pea and wheat (Dobres et al., 1987; Bringloe et al., 1995).

Flavodoxin is a well known small flavoprotein that has been isolated from a number of prokaryotes as well as from some algae. In some species, flavodoxin gene expression was found to be constitutive (Razquin et al., 1994), while in other organisms it is induced as a result of iron deficient conditions in the media. In the latter case, flavodoxin is believed to replace ferredoxin in vivo in those reactions which normally use ferredoxin (Mayhew and Ludwig, 1975). In *Scenedesmus vacuolatus*, flavodoxin effectively replaces ferredoxin in $NADP^+$ photoreduction (Peleato et al., 1994), ferredoxin-dependent nitrite reduction and ferredoxin-dependent glutamate synthase electron transfer (Vigara et al., 1998). Iron controls the expression of inducible flavodoxin at the level of transcription (Laudenbach et al., 1988; Razquin et al., 1994), and, in the case of the cyanobacteria, several Fur (Ferric uptake regulation) protein-binding sites have been found in the promoter sequences of the flavodoxin gene (Bes et al., 2001).

The current methods for quantifying flavodoxin and ferredoxin use HPLC (Doucette et al., 1996; Erdner and Anderson, 1999) or immunochemical methods such as non-quantitative Western blots (LaRoche et al., 1995, 1996; McKay et al., 1997, 1999), or immunoelectrodiffusion (Laurell, 1966; Inda and Peleato, 2002). HPLC methods achieve a linear response from 2 to 100 μg/ml for flavodoxin and from 1 to 100 μg/ml for ferredoxin (Doucette et al., 1996) in crude extracts. This method requires a considerable amount of cells and has similar limits of detection as immunoelectrodiffusion.

In this paper, we describe the development of two sandwich ELISA tests, one for flavodoxin and the other for ferredoxin. These ELISAs have been developed using *Scenedesmus vacuolatus* (former *Chlorella fusca* Shihira et Kraus 211-15) proteins and antibodies. We chose this green alga to avoid a wide range of cross-reactivity with proteins from other organisms, with different behaviour in the ferredoxin/flavodoxin interchange as response to iron starvation, and to facilitate the interpretation of the data. The *S. vacuolatus* ferredoxin and flavodoxin ELISAs were designed to test iron deficiency in fresh waters, but the methodology can be also reproduced for marine systems. In the latter case, it will be very important to find a suitable organism fitting the conditions of a well-typified ferredoxin/flavodoxin interchange and narrow antigen cross-reactivity.

2. Results

In the case of *S. vacuolatus*, the antisera raised against flavodoxin did not recognize ferredoxin and a similar result was obtained when anti-ferredoxin was tested against flavodoxin. Ferredoxins isolated from the cyanobacterium *Anabaena* sp. PCC7119 and from the marine diatom *Thalassiosira weissflogii* [a kind gift from Dr. D.L. Erdner (Woods Hole Oceanographic Institut)] did not cross-react with our anti-ferredoxin. Anti-flavodoxin (*S. vacuolatus*) did not recognize flavodoxin from *Anabaena* sp. PCC7119. The generated polyclonal antibodies did not cross-react with the corresponding proteins from the heterotrophic bacterium *Azotobacter vinelandii* nor did antibodies raised against flavodoxin and ferredoxin from cyanobacteria recognize the corresponding proteins from *S. vacuolatus*. Ferredoxin and flavodoxin from *Monoraphidium braunii*, a green alga, were recognized by the antibodies from *S. vacuolatus* (Inda and Peleato, 2002).

The purification of the polyclonal antibodies was performed using more stringent conditions than usual, because without 10% dioxane in the elution buffer, the antibodies did not elute. Crude extracts containing the total soluble protein were used as immunoadsorbent, after failure of more conventional methods, such as the use of pure proteins incubated with glutaraldehyde and polyacrylamide-agarose (Ternynck and Avrameas, 1989), or pure proteins incubated with agarose activated by cyanogen bromide (Dean et al., 1985).

The determined optimal concentration of the first antibody linked to the plate was 40 μg/ml for anti-ferredoxin and 60 μg/ml for anti-flavodoxin. The best blocking buffer was 3% bovine serum albumin, although 5% non-fat dry milk also gave good results. It is not advisable in our case to use detergents such as 0.05–0.2% Tween-20 as described currently in the literature. The optimal incubation time for standard samples was 2 h. Several concentrations of the second antibody were tested, and the optimal concentrations found for the peroxidase-labelled antibodies were 4.5 μg/ml for anti-ferredoxin, and 11 μg/ml for anti-flavodoxin. The optimal incubation time was also 2 h.

Fig. 1 shows the linearity of the response of the ELISA developed for flavodoxin, indicating that flavodoxin can

be determined between 30 and 300 ng/ml. Similar results were obtained using the ELISA for ferredoxin (Fig. 2), with a linear response in a range of 30 to 600 ng/ml. Detection below 30 ng was compromised by background noise, calculated using three times the standard deviation of the absorbances calculated for values in Figs. 1 and 2. Values over 300 ng/ml in the case of flavodoxin and 600 ng/ml for ferredoxin, gave a non-linear response due to saturation. In both cases, the detection limits are 30 ng/ml while HPLC/PDA and immunoelectrodiffusion methods remain at about 1 μg/ml for both proteins (Doucette et al., 1996; Inda and Peleato, 2002).

In order to control the accuracy of the ELISAs developed, samples and standards quantified by the ELISA were immunoquantified by immunoelectrodiffusion as described previously (Inda and Peleato, 2002). Table 1 shows the amount of flavodoxin and ferredoxin determined in crude extracts comparing both immunoelectrodiffusion and ELISA. The ELISAs were performed in the range of immunoelectrodiffusion limits, and the values obtained in both cases are very similar.

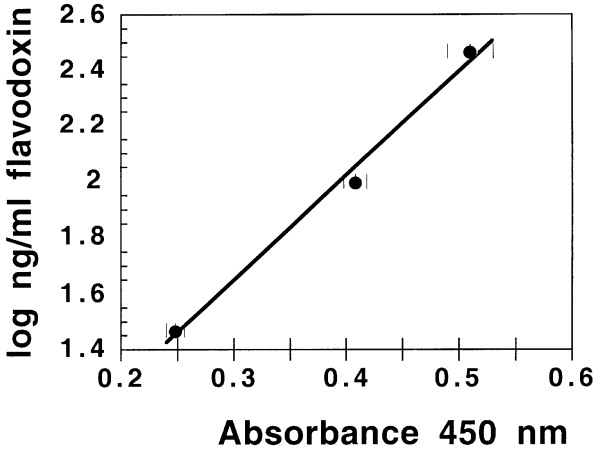

Fig. 1. ELISA quantification of *Scenedesmus vacuolatus* flavodoxin. Logarithmic representation of flavodoxin concentration (30, 100 and 300 ng/ml) versus absorbance at 450 nm. Values are the mean of four determinations.

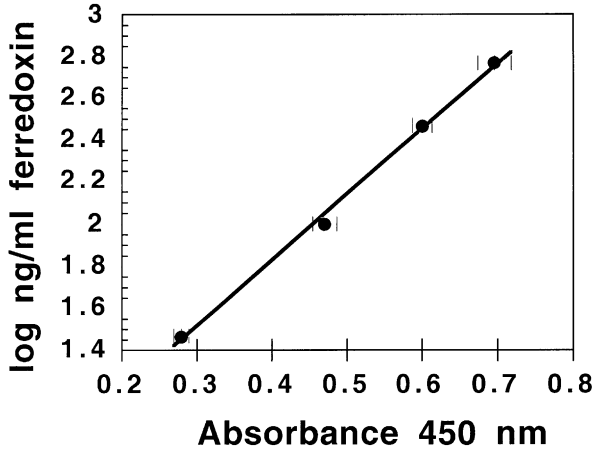

Fig. 2. ELISA quantification of *Scenedesmus vacuolatus* ferredoxin. Logarithmic representation of ferredoxin concentration (30, 100, 300 and 600 ng/ml) versus absorbance at 450 nm. Values are the mean of four determinations.

3. Discussion

Flavodoxin and ferredoxin have frequently been proposed as molecular markers for iron deficiency (Entsch et al., 1983; LaRoche et al., 1995; Doucette et al., 1996), but the applicability of the diagnosis is dependent on several factors, such as a suitable methodology, with the interpretation of field data being the main problem. It is important to take into account the methodology used for the quantification of the proteins due to its repercussion on data interpretation.

We are aware of the complexity of the interpretation of field data, a common problem with different connotations in the different methods used for quantification of ferredoxin and flavodoxin. Using HPLC, the over-all quantification of flavodoxin and ferredoxin of the organisms living in a community requires a complicated assessment. Whereas ELISA and all of the immunological methods quantify the ferredoxin or flavodoxin from one species or phylogenetically related ones, HPLC quantifies all the ferredoxins and flavodoxins present in the sample, since they show similar chromatographic behaviour. HPLC detects only holoproteins, whereas immunochemical methods detect both apoproteins and holoproteins. All data, total protein or holoprotein, must be interpreted from the physiological point of view, concerning the regulation of the expression of the protein in each organism (constitutive flavodoxin, no flavodoxin gene, iron deficiency inducible flavodoxin).

Since the response of the replacement of flavodoxin/ferredoxin presents extraordinary variability (Erdner et al., 1999), the possibility to typify the response in a well-studied model organism or group is going to simplify a very complex problem. In our case, immunoquantification of the flavodoxins and ferredoxins from green algae would be expected. We propose the use of a very ubiquitous organism in fresh waters (Fott and Novakova, 1969) to be used as a tool for the diagnosis of iron stress.

Table 1
Comparative study of the quantification of ferredoxin and flavodoxin using the ELISA developed in this work and immunoelectrodiffusion[a]

	ELISA (μg/ml)	Immunoelectrodiffusion (μg/ml)
Flavodoxin	15.9±1.3	13.0±1.8
Ferredoxin	21.3±5	18.1±3.5

[a] Iron deficient cells (1 μM iron in the culture media) were used for the quantification. Values are the mean±S.D. ($n=3$). Samples in the range of detection of immunoelectrodiffusion were diluted to fit in the ELISA range and tested as indicated in Experimental section.

The two ELISA tests are 30 times more sensitive than the current HPLC methods or quantification by immunoelectrodiffusion. The amount of sample necessary is very small when the ELISA is used, therefore limitation on sampling can be overcome. Components of the ELISA test can be prepared beforehand, stored at 4 °C for several months, transported, and developed without sophisticated equipment or specialized staff.

These tests have been developed for an organism from fresh waters, but this powerful technique can be extrapolated easily to marine environments, using proteins from model marine organisms, with well-typified responses to iron deficiency. Enrichment bioassays, an excellent approach to asses iron deficiency, can also use ELISAs to improve the quantification of ferredoxin and flavodoxin, since the method presented is rapid, specific and very sensitive.

4. Experimental

4.1. Organism. Culture media and condition

The green alga *Scenedesmus vacuolatus* [formerly *Chlorella fusca* (Shihira et Kraus 211-15)] (Hegewald and Schnepf, 1991; Kessler et al., 1997) was obtained from the University of Göttingen (Germany) culture collection. Cells were grown in batch cultures at 28 °C as described by Kessler and Czygan (1970). In order to purify the flavodoxin, iron-deficient cells were cultured using 0.18 μM iron, and to purify the ferredoxin, iron-sufficient cells were cultured using 18 μM iron.

4.2. Flavodoxin and ferredoxin purification

Flavodoxin for standards and antibody preparation, was isolated from iron-deficient cultures of *S. vacuolatus*, according to Peleato et al. (1994). Ferredoxin was purified from the iron-sufficient cells as described in Bes et al. (1999).

4.3. Protein quantification

Total protein quantification was performed by the Lowry method (Lowry et al., 1951). Pure ferredoxin was quantified using an extinction coefficient (420 nm) of 10.4 $mM^{-1}\,cm^{-1}$ (Bes et al., 1999), and for pure flavodoxin (462 nm), 10 $mM^{-1}\,cm^{-1}$ was used (Zumft and Spiller, 1971).

4.4. Generation of polyclonal antibodies

Antibodies were obtained from New Zealand white rabbits as described by Johnstone and Thorpe (1987). Rabbits were inoculated with 50 μg of pure protein with an equal volume of complete Freund coadjuvant. After 4 weeks, a second inoculation was undertaken with the same amount of protein and an equal volume of incomplete Freund coadjuvant. A week after the second inoculation, when the animal serum contained a high level of antibody, the rabbit was bled. Serum was separated from cells by centrifugation. Cross-reactivity was studied by the Ouchterlony (1949) technique.

4.5. Purification of antibodies

The antibodies, anti-flavodoxin and anti-ferredoxin, from the obtained antiserum were purified using immunoadsorbents. These imunoadsorbents were made using crude extracts from *S. vacuolatus* grown in different iron conditions. Protein extracts from iron-deficient cultures contain flavodoxin, whereas protein extract containing ferredoxin was obtained from cells grown in iron-sufficient conditions. These protein extracts were insolubilized using the method described by Avrameas and Ternynck (1969) modified by Lampreave (1978). The purification method is based on the specific interaction between antibodies and antigens on the immunoadsorbent. Crude serum (10 ml) obtained from rabbits were incubated in the column for 2 h. 0.01 M sodium phosphate pH 7.4 with 0.15 M NaCl (PBS) was used for washing the unbound material. Once the absorbance (280 nm) was below 0.02, the antibodies were eluted with 0.1 M glycine-HCl pH 2.8 with 0.4 M NaCl and 10% dioxane. The fractions were studied measuring the 280 nm absorbance and the obtained antibody pool was neutralized with 0.2 M bipotassium phosphate. Finally, the solution was concentrated to 5 mg/ml by ultrafiltration in Amicon cells. The last step was a dialysis against PBS. All of these operations were performed at 4 °C with precooled solutions, except the elution of the antibodies which was performed at 2 °C.

4.6. Preparation of peroxidase-conjugated antibodies

The preparation of the peroxidase conjugates were performed in a single step, as described previously (Ternynck and Avrameas, 1989). Dialysis of 1 ml of antibody (5 mg/ml) against 0.1 M potassium phosphate pH 6.8 was performed at 4 °C overnight. Horseradish peroxidase (10 mg; Sigma) was added and later, 50 μl of 1% glutaraldehyde in 0.1 M potassium phosphate pH 6.8 buffer. The solution was maintained at room temperature for 2 h; the reaction was stopped by adding 50 μl of 1 M glycine.

4.7. Development of the ELISAs

Although both ELISAs were developed separately, the procedure is the same. As solid support plates we used either Maxisorp (Nunc) or those from Universal Binding and Enhanced Binding (Labsystems).

The unlabelled antibody (anti-ferredoxin or flavodoxin) was added to the bottom of each well by adding

50 μl of antibody solution. This antibody solution had a concentration of 40 μg/ml anti-ferredoxin or 60 μg/ml of anti-flavodoxin in 0.1 M sodium carbonate/ sodium bicarbonate pH 9.6 buffer. The plates and the antibodies were incubated for 2 h at room temperature in a humid atmosphere. After that, the wells were washed three times with 400 μl PBS. The wells were filled to the top with blocking buffer, PBS with 3% (w/v) bovine albumin and 0.01% (w/v) sodium ethylmercurithiosalicylate. The plates were incubated overnight at room temperature in a humid atmosphere. The wells were washed three times with PBS (400 μl/ well), before adding 50 μl of the antigen (standards or samples) to the wells. Once the plates had been incubated for 2 h at room temperature in a humid atmosphere, the wells were washed three times with PBS. The labelled second antibody was added, with anti-flavodoxin at 4.5 μg/ml and anti-ferredoxin at 11 μg/ml. The reaction mixture was incubated for 2 h at room temperature in a humid atmosphere, and the wells were washed four times with 400 μl/well PBS. The formation of the coloured enzymatic product was started by adding 100 μl of peroxidase substrate (3,3′, 5,5′-tetramethylbencidine (k-blue, Neogen)) to the wells. The enzyme reaction should be stopped after 30 min by adding 100 μl of 1 M HCl. Absorbance at 450 nm of standards and samples was recorded using a Dynatech MR5000, and used for quantification. To determine the optimal reagent concentrations for the ELISA, serial dilution titration analyses were performed (Harlow and Lane, 1988).

4.8. Preparation of crude extracts

Cells were broken down using a Mini-Beadbeater from Biospec Products with 0.5 mm zirconium beads. 50 mM Tris-acetate pH 8, containing 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM EDTA and 10 mM PMSF (phenylmethylsulfonyl fluoride) were used as the extraction buffer.

4.9. Chlorophyll determination

Chlorophyll content was determined spectrophotometrically as described by MacKinney (1941).

4.10. Immunoelectrodiffusion

Ferredoxin and flavodoxin were immunoquantified, when indicated, using the procedure previously described by Inda and Peleato (2002).

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