Highly Efficient Purification of the Labile Plant Enzyme 5-Aminolevulinate Dehydratase (EC 4.2.1.24) by Means of Monoclonal Antibodies

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5-Aminolevulinate dehydratase (ALAD) from spinach (*Spinatia oleracea*) was isolated by affinity purification on an immunoabsorbens with a yield of 70 to 80% of the activity in the crude enzyme preparation. The enzyme eluted from the immunoabsorbens was pure as judged by polyacrylamide gel electrophoresis and is a hexamer with a subunit molecular weight of about 50000. Enzyme bound to the immunoabsorbens was able to synthesize porphobilinogen in a continuous manner. Owing to the lability of the enzyme and its low abundance in plant tissue, we have been unable to obtain similar yields of purified enzyme using classical purification procedures.

This highly efficient purification was made possible by using monoclonal antibodies as described by Köhler and Milstein (Nature **256,** 495 (1975)). The availability of monoclonal antibodies meant that it was not necessary to purify the enzyme to homogeneity by classical means in order to raise an antiserum specific for ALAD. Sixteen clones of cells producing antibodies against ALAD were selected. They all expressed a $\varkappa$ light chain but differed in the heavy chain class which was either γ_1 or γ_{2a}.

The availability of pure ALAD enzyme and of highly specific antibodies against the enzyme now enables us to answer questions concerning properties, localization, intercellular transport and evolution of ALAD. It is clear that the technique used and the questions asked are not restricted to ALAD.

Introduction

5-Aminolevulinate dehydratase (ALAD) from higher plants, which catalyzes the second step in chlorophyll and porphyrin biosynthesis has previously been purified about 300 to 500 fold using classical means (for a summary see [2]). However, a more thorough purification, which is required for subunit studies ect. is limited by a variety of difficulties encountered in the purification of many plant enzymes. Large amounts of plant material would be required owing to the low protein content of the tissues. The enzyme losses during such purifications are high and the final enzyme preparations are not only impure but also labile because of the low protein concentration in the final sample.

Most of these difficulties could be avoided using antibodies which selectively precipitate the enzyme from crude plant extracts. However, the preparation of a specific antiserum – and this is the crucial point – requires the prior availability of pure enzyme.

To overcome this obstacle, we applied the cell fusion technique of Köhler and Milstein [1] as modified by Hämmerling [3]. This technique is based on the immortalization of individual antibody producing cells by hybridization with myeloma cells. These hybrid cell lines which continuously secrete antibodies can be grown *in vitro* or as a tumor in mice and allow the isolation of large amounts of antibodies specific for a single determinant of an antigen.

By immunoabsorption on monoclonal antibodies which were prepared and selected according to this technique, the primary problem, the purification of ALAD, was solved. Moreover, the antibodies can be expected to be extremely useful in answering a variety of questions concerning the localization and the intracellular transport of ALAD as well as for taxonomic studies.

Abbreviations: ALA, 5-aminolevulinate; ALAD, 5-aminolevulinate dehydratase; BSA, bovine serum albumin; DPBS, Dulbecco's phosphate buffered saline (Seromed, München); ME, mercaptoethanol; PBG, porphobilinogen; PBS, phosphate buffered saline (Flow Labs., Meckenheim); Tris, tris(hydroxymethyl)-aminomethane.

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Materials and Methods

Cultivation of spinach

Spinach (Rup/Mona Lisa) was grown outdoors in the garden of the botanical institute and harvested

before shoots developed. Spinach grown in spring yielded the highest amounts of ALAD.

Prepurification of ALAD

ALAD was purified as described previously [4], but with minor modifications. The procedure consisted of the following steps: homogenisation of spinach in Tris solution containing ME (final pH about 8) and following centrifugation acetone and ammonium sulfate fractionation and chromatography on DEAE cellulose (DE 52, Whatman). Precipitation by ammonium sulfate was now omitted and chromatography on Sephacryl S 300 (100×2.6 cm) was added after chromatography on DEAE cellulose. In contrast to the DEAE cellulose used previously, DE 52 separated ALAD with only one activity peak. In all fractions the pH was maintained at pH 8.2 by Tris-HCl buffer (0.05 M) containing $MgCl_2$ (5 mM) and ME (5 mM). The enzyme preparation could be kept in liquid nitrogen without loss of activity.

Since the probability of getting sufficient amounts of specific antibodies increases with the purity of the antigen used to elicit them purification of ALAD was continued. The most active fractions from the Sephacryl column were pooled and concentrated by ultrafiltration. The concentrate of 0.5 kg of spinach was applied to 10 polyacrylamide gels (100×7 mm, 7.5% [5]), a volume of 100 µl per gel. The active discs were cut out and filled together with electrophoresis buffer into the upper compartment of an electrophoresis tube which was separated into two compartments by a small stopper of polyacrylamide gel. The lower, 0.5 ml compartment filled with the same buffer was sealed with a piece of ultrafilter membrane. The tube prepared in this way was inserted into the usual electrophoresis device and developed until all activity had accumulated in the lower compartment. 24 to 36 h at 3 mA were sufficient. The protein solution obtained by this procedure was removed from the lower compartment with a syringe and was used for immunization. The number of proteins was reduced to 3 bands (see Fig. 1 a).

Immunization of mice

Mice of the inbred strain BALB/c were injected i.p. with 100 µl per animal of the ALAD preparation emulsified with an equal volume of Freund's complete adjuvant. After three weeks, the mice received an additional i.p. injection of 50 µg ALAD preparation in Freund's incomplete adjuvant in a total volume of 200 µl per animal. Fourteen days later the mice got a last challenge with 25 µg of the antigen in saline. Half of the antigen was applied i.p. the other half i.v. Two days thereafter the spleen was isolated and the spleen lymphocytes fused with myeloma cells.

Cell fusion

The myeloma cells for fusion were the BALB/c strain-derived tumor X63-Ag 8.653 [6]. These cells were permanently cultured *in vitro* under appropriate culture conditions, and centrifuged and suspended in DPBS before fusion.

Lymphocytes were isolated by gently grinding the spleen of immunized mice in DPBS and removing the cell debris.

We followed the fusion technique used by Hämmerling ([3] and personal communication). Lymphocytes and tumor cells were mixed at a ratio of 1:10 at room temperature, centrifuged and resuspended in 1 ml of prewarmed (37 °C) polyethylene glycol solution. After 1 min of gentle shaking the suspension was slowly diluted by dropwise addition of 6 ml of DPBS. The cells were pelleted again and resuspended in prewarmed selective HAT culture medium. The cell suspension was then distributed on multiwell tissue culture plates (Costar 3524, Cambridge, Mass.). The final concentration was about 5×10^5 cells per well in 1.5 ml.

Only cells formed by the fusion of a lymphocyte with a tumor cell will grow on the selective HAT medium. Lymphocytes do not grow continuously *in vitro* and X63-Ag 8.653 cells are killed because of their enzymatic deficiency (hypoxanthine guanosyl phosphoribosyl transferase) and the blockage of *de novo* synthesis of purines and pyrimidines by aminopterin (inhibiting dihydrofolate reductase).

Selection of cells producing antibodies against ALAD

Cells producing antibodies directed against ALAD were detected by the following procedure: Wells of microtiter plates (Cooke Laboratory Products) were coated with sheep anti-mouse immunoglobulin (2 µg/ml DPBS). 50 µl were applied to each well. After incubation over night at 4 °C the supernatants were flicked out. Remaining free binding sites of the surface of the well were saturated with 2% BSA in

DPBS for 2 h at room temperature. After washings with PBS 50 µl of the supernatants from wells in which cells grew were added and the plates stored at 4 °C over night. After extensive washings, 50 µl of the antigen solution was added and the plates again stored at 4 °C over night. After 6 washings to remove all non-bound ALAD, the wells were supplied with 100 µl of the ingredients for an ALAD enzyme assay (0.2 mg ALA/ml of Tris-HCl pH 8.2 containing 5 mM of $MgCl_2$ and ME). PBG was allowed to form over night at 25 °C. After addition of 100 µl of Ehrlich's reagent [7] containing $HgCl_2$ a red stain disclosed PBG and thus indicated wells containing antibodies against ALAD.

Cells producing positive supernatants were recloned. For the first screening clones synthesizing antibodies against the components of the ALAD preparation were identified by means of an enzyme-linkes immunosorbent assay (ELISA) as described in the literature [6, 8].

The heavy and light chain composition of ALAD-binding antibodies was determined by a similar assay. The necessary antibodies were a generous gift from J. Kearney and C. Müller (laboratory of Prof. Rajewsky, Cologne).

Cell cloning

The cells of positive wells were subcloned according to the limiting dilution technique [9]. In order to avoid continuous culture of subcloned cells, the cells were frozen in liquid nitrogen and stored at −75 °C.

Preparation of large amounts of antibodies

Cells from positive clones were injected i.p. into Pristane-pretreated BALB/c mice (see [10]). This procedure allows to obtain large amounts of ascites fluid containing 2 to 20 mg of antibody per ml. Antibodies were purified from ascites fluid by ammonium sulfate precipitation and DEAE cellulose chromatography (see [11]).

Preparation of Sepharose bound antibodies

Sepharose bound antibodies were prepared by the CNBr method of March *et al.* [12].

Culture conditions and media

Myeloma cells of the strain X63-Ag 8.653 were grown at 37 °C under an atmosphere enriched with 10% of CO_2. The culture medium was RPMI 1640 (Seromed, München) supplemented with 5×10^{-5} M of ME, 10% of fetal calf serum, 100 units/ml of penicillin and 100 µg/ml streptomycin. In addition to the components of the culture medium the selective HAT medium (see [13]) contained hypoxanthine (0.1 M), aminopterin (0.4 µM) and thymidine (16 µM).

The medium in which cells were frozen consisted of DPBS containing 20% of fetal calf serum and 15% of dimethylsulfoxide. Cell fusion was performed with 42% of polyethylene glycol (mol. weight 2000 to 4000) in DPBS containing 15% of dimethylsulfoxide [3].

Results

Antibody producing clones

Two to three weeks after seeding the cell fusion mixture into the wells of tissue culture plates about 150 out of 360 wells of tissue culture plates about As detected by the techniques described under methods the supernatants of 35 cell colonies contained antibodies against the enriched ALAD preparation. Sixteen colonies out of these 35 colonies produced antibodies directed against ALAD. The ALAD positive cells were subcloned and samples injected into mice in order to obtain larger amounts of antibodies.

Because of the method applied antibodies binding to the active center of the enzyme may have escaped detection.

The antibodies synthesized by the ALAD positive cell colonies all have a $\varkappa$ light chain. The heavy chain is γ_1 in 12 colonies and γ_{2a} in 4 colonies. More about the characteristics of these hybrid cell lines will be published in a study on the evolutionary relations of ALAD of different origins (Z. Naturforsch.).

Purification of ALAD

Antibodies from one randomly selected cell colony (No. 153) were bound to Sepharose. The solubilized and dialyzed acetone precipitate from 1 kg of spinach prepared according to the first step of the prepurification procedure (see Methods) was incubated with gentle shaking with 7 ml of Sepharose-bound antibodies for 24 h at 4 °C. One ml of Sepharose contained about 1.4 mg of antibodies.

The slurry was poured into a column (2 cm in diameter) to separate ALAD from the bulk proteins. Effluents which still contained activity were again passed through the column which was finally washed with about 200 volumes of buffer (5 mM Tris-HCl pH 8.2, containing 5 mM of $MgCl_2$ and ME). ALAD was then eluted with glycine-HCl pH 3 (0.17 M). The eluate was immediately brought to pH 8.2 with 0.5 M Tris-HCl and supplemented with 5 mM of $MgCl_2$ and ME. Approximately 80% of the activity applied to the column was regained. No experiments were performed to optimize the elution procedure further.

The stained electropherogram of the eluted protein is shown on Figure 1 b. Only a single band is visible and this band comigrates with the band of ALAD activity on the gel. For comparison, the profile of proteins present in the enzyme preparation before the antibody-mediated separation is given (Fig. 1 c). On this gel, the ALAD band is obscured by high amounts of other proteins.

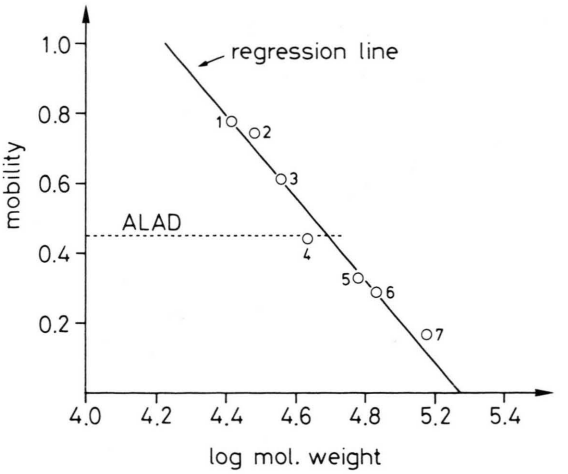

Fig. 2. Determination of the subunit molecular weight of ALAD on SDS polyacrylamide gels. The figure shows one experiment out of a series of 4 similar ones. Calibration proteins (Pharmacia) and their molecular weights: 1) chymotrypsinogen, 25700; 2) carbonic anhydrase, 30000; 3) lactate dehydrogenase, 36000; 4) ovalbumin, 43000; 5) catalase, 60000; 6) albumin, 67000; 7) phosphorylase b, 94000 (gels according to [18]).

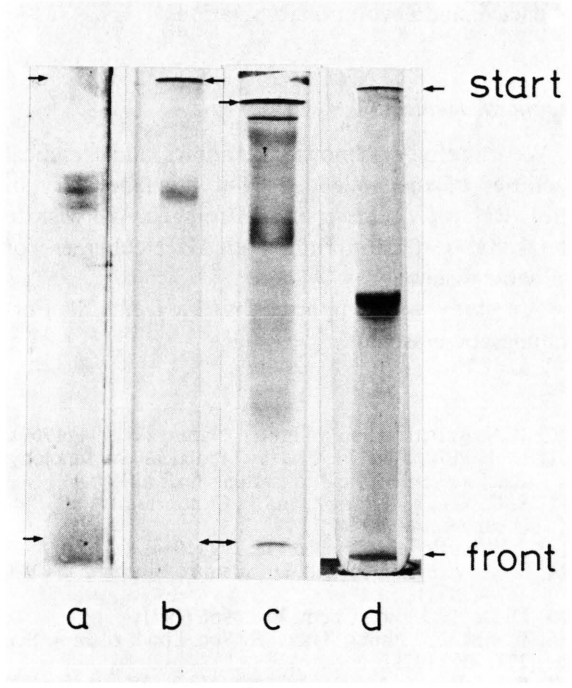

Fig. 1. Steps in ALAD purification as analyzed by polyacrylamide gel electrophoresis. a) ALAD preparation injected into mice; b) ALAD purified by means of Sepharose-bound monoclonal antibodies; c) ALAD preparation applied to the column of Sepharose-bound monoclonal antibodies; d) Purified ALAD on a SDS gel.

According to the amount of ALAD protein eluted from the column and the estimated losses during prepurification, one kg of spinach contains about 3 mg of ALAD enzyme.

Analysis of the purified protein on SDS polyacrylamide gels shows a strong band in the region of 50000 daltons (Figs. 1 d and 2).

The evaluation of 4 experiments by means of regression lines according to the principle of least squares revealed subunit molecular weights of ALAD of 49500 with deviations of about 500 daltons. If ovalbumin which badly fits the regression line (see Fig. 2) is omitted from the calculations, the subunit molecular weight is 50500. Because density centrifugation of spinach ALAD had revealed a native molecular weight of 324000 ± 14000 [4], it appears that ALAD from spinach is a hexamer rather than an octamer.

Six subunits correspond with the number of subunits of ALADs from *Rhodopseudomonas spheroides* [14] and mouse [15]. Bovine ALAD was found to consist of 8 subunits [16]. In contrast to the latter enzyme, the enzyme from *R. spheroides* showed antigenic similarities with the spinach enzyme. The supernatant of one cell line out of the 16 isolated ones, which produced antibodies against spinach

ALAD, was also positive against ALAD from *R. spheroides.*

A second band on SDS polyacrylamide gels indicating a molecular weight of about 45000 daltons appeared if the gels were overloaded (see Fig. 1 d) with the purified protein. The intensity of this band was about 5% of the intensity of the heavy band.

Continuous synthesis of PBG

ALAD, immobilized on the immunosorbent can synthesize PBG in a continuous manner. Using a column with 100 µg of bound ALAD which was perfused with 12.5 ml of substrate solution per h (2 mg ALA/ml in 0.1 M Tris-HCl pH 8.2, containing 5 mM of ME and $MgCl_2$) at 25 °C, the turnover rate of ALA was 62%. The bound enzyme appeared to be more stable than the enzyme in solution.

Concluding remarks

The main outcome of the present study is that the method applied is superior to all conventional enzyme purification procedures. ALAD protein is obtained pure and the enzyme product PBG can be synthesized in a continuous process. Optimization of the elution buffer system might even lower the losses in enzyme activity which occurred during our experiments. We are sure that the pains prior to the simple purification procedure are much less time consuming than the efforts to elaborate a traditional purification system to the point where it is equally efficient.

It is obvious that the methods used for enzyme purification and product synthesis are also applicable to a great variety of other plant enzymes which are of scientific or biotechnological interest and which are not available in the pure form because of their lability and their low concentration in plant tissues. The methods have been used to solve problems concerning animal physiology (see [17]). Monoclonal antibodies will enliven research in all the fields of plant physiology and biochemistry which are limited by the impossibility to obtain specific antisera. As regards our fields of investigation, we may list discrimination between isoenzymes, immunoprecipitation of enzymes *in statu nascendi* and localization and characterization of enzymes in different cell compartments. If specific antisera are useful in these studies, they may be obtained *via* the pure enzymes. Another very important point is the possibility of using monoclonal antibodies in taxonomic studies. Evolutionary trees based on the antigenic determinants of an enzyme or protein may be established. We started such studies with ALAD from origins as different as from bacteria, algae, mosses, ferns, gymnosperms and angiosperms. The results supplement morphological, chemical and sequence data on evolutionary relations.

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