Comparison of Different Methods for the Determination of Phenylalanine Hydroxylase Activity in Rat Liver and *Euglena gracilis*

Rita M. Fink and Erich F. Elstner

Institut für Botanik und Mikrobiologie, Technische Universität München, Arcisstr. 21, D-8000 München 2, Bundesrepublik Deutschland

Z. Naturforsch. **39c,** 728–733 (1984); received January 27/April 14, 1984

Phenylalanine Hydroxylase, Rat Liver, *Euglena gracilis*, Tyrosine Determination

Three different methods for the determination of phenylalanine hydroxylase activity have been compared:

a) Differential photometric assay of the increase in tyrosine concentration in the presence of phenylalanine;
b) Product separation by thin layer chromatography and scintillation counting of the [^{14}C]tyrosine formed;
c) HPLC separation and spectrofluorometric quantification of derivatized amino acids.

A comparison of the activities of phenylalanine hydroxylase in rat liver and *Euglena gracilis* clearly showed that only rat liver contains this enzymic activity as shown by methods b) and c) although pseudo-activity of *Euglena gracilis* preparations was found during the spectrophotometric test a).

The HPLC method proved to be the fastest, most reliable and convenient method for direct tyrosine determination and thus for measuring phenylalanine hydroxylase activity.

Introduction

The enzyme phenylalanine hydroxylase (EC. 1.14.16.1) (PAH) catalyses the hydroxylation of phenylalanine and was first isolated from rat liver by Kaufman [1].

Further extensive studies on the mechanism of function and distribution of this enzymic activity by several groups (Kaufman [2, 3], Ayling [4], Bailey [5], Hasegawa [6], Parniak [7], Shiman [8, 9], Lazarus [10, 11], Gottschall [12]) have shown that this mixed function oxygenase has a requirement for reduced pteridine cofactor. In mammalian tissues, especially in liver, tetrahydrobiopterin seems to be the natural cofactor. In contrast to animals, higher plants do not seem to contain this enzyme (Kirst [13]).

Paur [14] has recently shown that the phytoflagellate *Euglena gracilis* which exhibits both animal and plant type metabolism can convert phenylalanine to tyrosine in the dark (animal-type) as well as cinnamic acid into coumaric acid in the light (plant-type).

In the above report, isolated PAH from *Euglena gracilis* has also been demonstrated to be dependent on reduced pteridines. The comparison of various types of pteridines showed that reduced "euglenapterins" were the most active cofactors representing the lowest K_M-values.

These euglenapterins were isolated from *Euglena gracilis* first by Elstner and Heupel [15] and identified as 2-dimethylamino-6-(L-threo)-trihydroxypropyl-4-oxo-3,4-dihydro-pteridine and the corresponding 3′-mono-phosphate and 2′,3′-cyclophosphate by Böhme *et al.* [16].

In contrast to the findings of Paur [14] are recent reports by Byng *et al.* [17] who showed that *Euglena gracilis* synthesizes tyrosine exclusively *via* the arogenate pathway using prephenate as precursor.

In order to clarify the situation in *Euglena gracilis*, we used three different methods of tyrosine determination for the measurement of potential phenylalanine hydroxylase activity and compared the results with the activity of phenylalanine hydroxylase preparations from rat liver.

Abbreviations: PAH, phenylalanine hydroxylase; EC. 1.14.16.1; RL-PAH, rat liver phenylalanine hydroxylase; "E-PAH", *Euglena* phenylalanine hydroxylase; CAT, catalase; DTT, dithiothreitol; OPA, *ortho*-phthaldialdehyde; RP-HPLC, reversed phase high pressure liquid chromatography.

Reprint requests to Dr. Rita Fink, Strahlenbiologisches Institut der Universität München, Schillerstr. 42, D-8000 München 2.

0341-0382/84/0700-0728 $ 01.30/0

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

Experimental procedures

Materials

Dithiothreitol, *o*-phthaldialdehyde, 6,7-dimethyltetrahydropterin were purchased from Sigma; catalase from Boehringer, Mannheim; L-[U-^{14}C]-phenylalanine (496 mCi/mmol) from Amersham Buchler, phenyl-Sepharose CL-4B from Pharmacia; cation exchange resin AG 50 W-X 8, 200–800 mesh, H^+-form and Chelex 100 from BioRad; methanol, HPLC grade from Rathburn Chemicals.

TLC cellulose plates (20 × 20 cm, 0.25 mm thickness) and all other chemicals (analytical grade) were obtained from Merck, Darmstadt.

Methods

Preparation of phenylalanine hydroxylase from rat liver (Wistar rats) and "PAH" from Euglena gracilis:

RL-PAH was prepared by hydrophobic affinity chromatography on phenyl-Sepharose according to the method of Shiman [8] as modified by Gottschall *et al.* [12].

The preparation of "PAH" from *Euglena gracilis* was performed analogously to the rat liver PAH preparation. Photoautotrophically grown *Euglena gracilis* cultures (5 days) were centrifuged from the culture medium (10 min, 4 °C at 2300 × *g*) and washed with buffer containing 0.03 M Tris/HCl pH 7.25 and 50 µM EDTA. After recentrifugation and carefully removing the supernatant, the *Euglena* cell pellet was mixed with a minimum (ca. 15 ml) of homogenisation buffer [12] containing 80 mM phenylalanine and treated with a French press at 1260 psi at room temperature.

The degree of homogenisation was controlled microscopically. Similarly to the rat liver enzyme preparation, the *Euglena* homogenate was brought to final volume of 4 ml homogenisation buffer per g fresh weight of *Euglena* cells. This *Euglena* homogenate was centrifuged at 4 °C for 45 min at 48000 × *g*. For activation, the supernatant was incubated at 25 °C for 10 min, then the activated *Euglena* extract was also made 0.1% in Tween 80, 2.0 mM in DTT and 0.3 mM in ferrous ammonium sulfate before applying the extract to the first phenyl-Sepharose column.

The washing procedure as well as the elution step were performed in the same manner as described for RL-PAH [12]. Fractions from the first column containing spectrophotometrically detectable "E-PAH" activities (already eluted by the last wash buffer = buffer B) were applied in reversed order to a second smaller column of phenyl-Sepharose in order to obtain higher purification of our preparations.

Buffer B contained 0.03 M Tris/HCl pH 7.25, 50 µM EDTA, 10 mM phenylalanine, 15% glycerol (V/V) and 0.037% Tween 80 (V/V) [8]. The second column had 10% of the bed volume of the first column and was equilibrated with 10 bed volumes elution buffer (composition identical with buffer B but without phenylalanine).

Enzyme activity determination

PAH activity was assayed by three different methods for tyrosine detection

a) indirect photometric tyrosine determinations were performed by the method of Shiman *et al.* [8]: 1.0 ml assay mixture contained:

0.1 M potassium phosphate buffer pH 6.8 which was passed through a Chelex 100 column, 1 mM phenylalanine, 65 µg catalase, PAH-sample, 6 mM dithiothreitol and 60 µM 6,7-dimethyltetrahydropterin (instead of 6-methyltetrahydropterin in the original Shiman test system). The rates of tyrosine formation (product formation) were monitored at $\lambda = 275$ nm. This assay mixture was also used in methods b) and c) for direct tyrosine determination.

b) Use of [^{14}C]phenylalanine for the determination of PAH activity:

In addtion to 1 mM unlabelled phenylalanine, the standard assay mixture (see section a)) contained 2 µCi ^{14}C-labelled phenylalanine. For PAH activity determination, the sample tubes were incubated in a water bath at 25 °C for 45 min in the dark. In order to optimise the following product separation by thin layer chromatography, a pre-purification step as described by Lehmann *et al.* [18] was performed. Accordingly, the acidified assay mixtures were passed through small cation exchange columns (1.0 ml; cation exchange resin AG 50 W-X 800, 200–800 mesh, H^+-form).

Eluted fractions containing ^{14}C-labelled compounds were collected and evaporated to dryness at 30 °C using a rotavapor. After dissolving the residue of each sample in 100 µl 1 M aqueous

ammonia, an aliquot of the solution (25 µl) was applied to TLC cellulose plates and chromatographed in a mobile phase consisting of CCl_3H: CH_3OH: NH_3: H_2O = 26:14:4:1 (V/V/V/V). The cochromatographed standard solution of phenylalanine and tyrosine was visualized by the ninhydrin reaction and showed R_F-values of 0.57 for phenylalanine and 0.26 for tyrosine. The parallel running ^{14}C-labelled phenylalanine and tyrosine zones were quantified by scintillation counting.

c) HPLC separation and spectrofluorometric quantification of derivatized amino acids:

Apparatus

RP HPLC analyses were performed using Beckman gradient equipment consisting of two solvent delivery systems (Beckman, Model 112), a gradient former (organizer 340), a controller system and an injection valve with a 5 µl loop. Separation of OPA-amino acids were carried out at room temperature using a 5 µm Ultrasphere ODS-column (Altex, 250 mm × 4.6 mm i.d.).

Column effluents were monitored with a variable wavelength fluorescence spectromonitor (Modell RF-530, Shimadzu). The excitation wavelength was set at 360 nm, the emission wavelength at 455 nm. The integration of the HPLC chromatograms was accomplished using an Altex plotter-integrator Model C-R 1 A.

The amino acid derivatization with *o*-phthaldialdehyde and the gradient elution of the OPA-derivatives were performed according to the method described by Bober, Fa. Beckman [19].

Mobile phases

A) 0.05 M Na-acetate buffer pH 6.8 : methanol : tetrahydrofuran = 80:19:1 (V/V/V).
B) methanol : 0.05 M Na-acetate buffer pH 6.8 = 80:20 (V/V).

Flow rate: 1 ml/min.

The gradient conditions used were shown in Fig. 1.

Derivatisation reagent

50 mg of OPA were dissolved in 1 ml methanol. After adding 40 µl 2-mercaptoethanol, the solution was adjusted to a final volume of 10 ml with 0.2 Na-tetraborate buffer pH 9.5 [19].

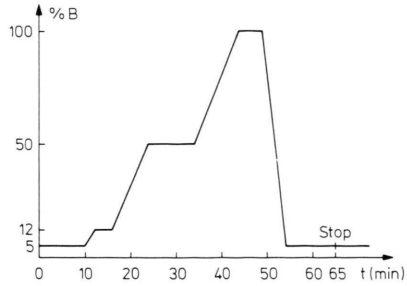

Fig. 1. Gradient program for RP-HPLC separation of amino acid OPA derivatives. Mobile phases: A) 0.05 M Na-acetate buffer pH 6.8 : methanol : tetrahydrofuran = 80:19:1 (V/V/V). B) methanol: 0.05 M Na-acetate buffer pH 6.8 = 80:20 (V/V). Flow rate: 1 ml/min.

Derivatisation procedure

20 µl deproteinized supernatant of the assay mixture (see point a)) were reacted with 40 µl derivatisation reagent at room temperature. After 1 min, about 40 µl of this reaction mixture was used to flush and fill the 5 µl sample loop for injection onto the column.

Standard solution

The calibration standard composed of alanine, serine, tyrosine and phenylalanine dissolved in 0.1 N HCl, contained in 5 µl derivatised and injected solution 20.8 pmol per amino acid.

d) Protein determination

The protein content was determined by a modified method of Lowry using Folin-Ciocalteus reagent [20].

e) *Culture of Euglena gracilis*

Euglena gracilis, strain Z was obtained from "Sammlung von Algenkulturen des pflanzenphysiologischen Instituts der Universität Göttingen".

Cells were grown photoautotrophically on a medium described by the above Institute in a Kniese algae culture thermostat.

Results

1) Photometric determination of PAH activity

In order to test photoautotrophically grown *Euglena gracilis* cultures for their PAH content, *Euglena gracilis* homogenates were treated in a similar manner to rat liver homogenates.

In contrast to rat liver tissue, spectrophotometrically active "E-PAH" fractions were already obtained by the last wash buffer B. PAH activity from rat liver was only detectable in the effluent from the elution buffer.

Photometrically measureable PAH activity in rat liver was dependent on eluted protein, the substrate phenylalanine and the reduced cofactor (data not shown), in agreement with [8] and [12].

The rate of conversion by RL-PAH is about 20% of the substrate phenylalanine (1 mM) with 60 μM 6,7-dimethyltetrahydropterin and 25 μg enzyme in 10 min.

The photometrically active fraction from the *Euglena* extracts was also dependent on the reduced pteridine cofactor and on the eluted protein, but not on the substrate phenylalanine. We first speculated that this might be due to the fact that the eluting wash buffer B was 10 mM in phenylalanine (Table I).

As shown in Fig. 2 the (boiling sensitive) rat liver enzyme was dependent on the presence of catalase. This dependence was manifested after two minutes of reaction. In contrast, the absorbance change measured in the presence of (heat stable!) *Euglena* PAH preparations was approximately half in the presence of catalase.

2) *Radioactive determination of PAH activities*

In order to verify the abnormalities of the PAH activities in *Euglena* preparation reported in section 1, we monitored the *Euglena* preparation in comparison to the corresponding rat liver enzyme by mean of TLC separation and scintillation counting of the substrate phenylalanine and its product, tyrosine.

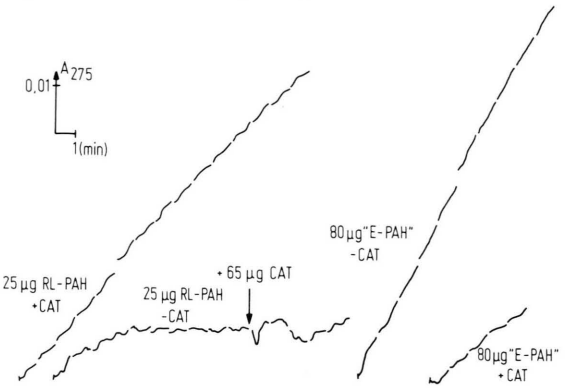

Fig. 2. Comparison of photometrically determined activities of RL-PAH and "E-PAH": effects of catalase.

As shown in Table II, freshly prepared *Euglena* preparations did not exhibit any tyrosine producing activities. In contrast, the rat liver enzyme converted approximately 18% of the initial substrate phenylalanine during the 45 min of incubation, whereas in the absence of catalase approximately 4% product formation (with respect to the blank) was observed.

3) *HPLC analysis of PAH activities*

In order to further investigate PAH activity in *Euglena gracilis* preparations, we used RP HPLC separation and fluorometric monitoring of amino acid derivatives. As shown in Table III, the *Euglena* preparations in comparison to the controls were not active in converting phenylalanine to tyrosine. The rat liver preparation in this test showed activity comparable to that observed in the radioactive test.

Discussion

The present paper deals with two major problems:

a) comparison of the established methods for phenylalanine as well as tyrosine analysis in

Table I. Photometric determination of the cofactor dependence of *Euglena gracilis* enzyme preparation.

Omission	Additions	nmol "TYR" ml min mg protein	
		1.–2. min	3.–7. min
none	40 μg E + PHE + DTT + PtH_4	58	77
– PHE	40 μg E – PHE + DTT + PtH_4	53	55
– PtH_4	80 μg E + PHE + DTT – PtH_4	0	0
– "E-PAH"	0 μg E + PHE + DTT + PtH_4	0	0

Assay mixture was as described under Materials and Methods, section a), but without catalase. Symbols: E = "*Euglena* phenylalanine hydroxylase"; PHE = phenylalanine; DTT = dithiothreitol; PtH_4 = 6,7-dimethyltetrahydropterin; TYR = tyrosine.

Table II. TLC separation of ^{14}C-labelled PHE and TYR after incubation with rat liver – or *Euglena gracilis* preparations.

Sample	cpm eluted total	cpm in PHE-zone	cpm in TYR-zone	nmol PHE recovered (= 100%)	nmol TYR formed	% conversion
none + CAT	577 500	561 700	2 400	650	3	0.4
+ "E-PAH" + CAT	595 700	579 200	2 400	680	3	0.4
+ "E-PAH" – CAT	513 100	495 200	2 100	570	2	0.3
+ RL-PAH + CAT	520 400	426 500	77 900	490	90	18.0
+ RL-PAH – CAT	573 300	550 700	5 700	640	7	1.1

Reaction conditions as described under Materials and Methods, section b). The reaction mixture contained 70 µg rat liver phenylalanine hydroxylase (RL-PAH) or 70 µg "*Euglena* phenylalanine hydroxylase" ("E-PAH"), respectively. CAT = 65 µg catalase.

Table III. RP-HPLC separation and fluorometric determination of PHE and TYR after incubation with rat liver and *Euglena gracilis* preparations and after derivatisation with *o*-phthaldialdehyde.

Sample	TYR $\frac{\mu g}{ml}$	PHE $\frac{\mu g}{ml} = 100\%$	% TYR
none + CAT + PHE + PtH_4	0.1	190	0.1
none – CAT + PHE + PtH_4	0.2	120	0.2
+ "E-PAH" – CAT + PHE – PtH_4	0.2	150	0.1
+ "E-PAH" – CAT + PHE + PtH_4	0.2	150	0.1
+ "E-PAH" – CAT – PHE + PtH_4	0.2	75	0.3
+ "E-PAH" + CAT + PHE + PtH_4	0.5	355	0.1
+ RL-PAH + CAT + PHE + PtH_4	38.5	190	20.6
+ RL-PAH – CAT + PHE + PtH_4	1.8	170	1.1

Reaction conditions as described under Materials and Methods, section c). The reaction mixtures contained 70 µg rat liver phenylalanine hydroxylase (RL-PAH) or 70 µg "*Euglena* phenylalanine hydroxylase" ("E-PAH"), respectively. Symbols as described in Tables I and II.

respect of the convenience and reliability of the test systems;

b) the question whether the phytoflagellate *Euglena gracilis* which is able to grow both autotrophically and heterotrophically synthesizes tyrosine from phenylalanine *via* the catalysis of the enzyme phenylalanine hydroxylase.

Since *Euglena gracilis* contains a new type of N-dimethyl-substituted pteridine, called euglenapterin (Böhme *et al.* [16]), the presence of such an enzyme might be indicated. Preliminary indications that such an enzyme might exist (Paur *et al.* [14]) are in contradiction of a subsequent report by Byng *et al.* [17]. This enzyme if it existed, was supposed to have novel requirements as far as the stereochemistry of pteridine cofactors is concerned: dimethyl substitution of the pteridine cofactor at the 2-amino position renders these factors inactive in the rat liver system (Kaufman [3]).

The results clearly show that the spectrophotometric monitoring of tyrosine in the generally applied test system is not reliable. A preparation from *Euglena gracilis* is highly active in this test system although no product can be identified by either the ^{14}C- or the HPLC method. The reason for this artificial absorbance increase is probably due to a non-proteinaceous (heat stable) factor in a corresponding preparation from *Euglena gracilis* extracts which has not been further identified. This factor mimics an enzymic activity which can lead to serious misinterpretations if not checked back by one of the direct methods for product quantification.

Comparing the ^{14}C-method with the HPLC method, the HPLC method clearly bears several advantages. Besides not having to deal with radioactive handling and disposal of waste, the HPLC method is much less time consuming and less prone

to experimental errors and losses of product yields. Both ^{14}C and HPLC methods are in good agreement as far as the total yields of conversion of phenylalanine to tyrosine are concerned.

In contrast to previous preliminary reports by Paur *et al.* [14], our results indicate that no real PAH activity is present in *Euglena gracilis* under the described preparation- and test conditions.

All applied methods show unequivocally that the rat liver system in addition to the usual substrate and cofactor requirements is dependent on the presence of catalase (as already shown by Kaufman [3] and other groups) in order to maintain the rate of conversion for times longer than one minute.

The mechanism of protection by catalase from a suicidal side effect during catalysis is under investigation and will be reported elsewhere.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft. We wish to thank H. Bober, Fa. Beckman Instruments for the introduction to the HPLC method and for discussions, and Dr. Nohl for providing the rat liver preparations.

We also thank Dr. R. J. Youngman for reviewing the manuscript.

[1] S. Kaufman, Methods in Enzymology **5,** 809 (1962).
[2] S. Kaufman, in "Chemistry and Biology of Pteridines", ed. Pfleiderer, W., pp. 291–304, Walter de Gruyter, Berlin, New York 1975.
[3] S. Kaufman and D. B. Fisher, in "Molecular Mechanisms of Oxygen Activation", ed. O. Hayaishi, pp. 285–369, Academic Press, London, New York 1974.
[4] J. E. Ayling and G. D. Helfand, in [2], pp. 305–319 (1975).
[5] S. W. Bailey, S. T. Weintraub, S. M. Hamilton, and J. E. Ayling, J. Biol. Chem. **257,** 8253 (1982).
[6] H. Hasegawa and S. Kaufman, J. Biol. Chem. **257,** 3084 (1982).
[7] M. P. Parniak and S. Kaufman, J. Biol. Chem. **256,** 6876 (1981).
[8] R. Shiman, D. W. Gray, and A. Pater, J. Biol. Chem. **254,** 11 300 (1979).
[9] R. Shiman and D. W. Gray, J. Biol. Chem. **255,** 4793 (1980).
[10] R. A. Lazarus, R. F. Dietrich, D. E. Wallick, and S. J. Benkovic, Biochemistry **20,** 6834 (1981).
[11] R. A. Lazarus, C. W. De Brosse, and S. J. Benkovic, J. Amer. Chem. Soc. **104,** 6869 (1982).
[12] D. W. Gottschall, R. F. Dietrich, S. J. Benkovic, and R. Shiman, J. Biol. Chem. **257,** 845 (1982).
[13] G. O. Kirst, Ph. D. Thesis (1971), TH Darmstadt.
[14] E. Paur, E. Lengfelder, and E. F. Elstner, in "Endocytobiology, Endocytosymbiosis and Cell Biology", eds. W. Schwemmler and H. E. A. Schwenk, **Vol. 1,** pp. 335–345, Walter de Gruyter, Berlin, New York 1980.
[15] E. F. Elstner and A. Heupel, Arch. Biochem. Biophys. **173,** 614 (1976).
[16] M. Böhme, W. Pfleiderer, E. F. Elstner, and W. J. Richter, Angew. Chemie **92,** 474 (1980).
[17] G. S. Byng, J. Whitacker, L. C. Shapiro, and R. A. Jensen, Mol. Cell. Biol. **1,** 426 (1981).
[18] W. D. Lehmann, N. Theobald, and H. C. Heinrich, Biomedic. Mass Spectrometry **8,** 598 (1981).
[19] Beckman Instruments, Fachgebiet Chromatographie, Untersuchungsbericht Nr. 1–7/82 (Bober.), Ref. J. Gibson, ETR. 81.023.
[20] E. F. Hartree, Anal. Biochem. **48,** 422 (1972).