S-ADENOSINE-L-HOMOCYSTEINE HYDROLASE FROM *Nicotiana tabacum* L.: ISOLATION AND PROPERTIES*

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S-Adenosyl-L-homocysteine hydrolase (E.C. 3.3.1.1) (SAH hydrolase) from an explantate culture of *Nicotiana tabacum* L. was purified to homogeneity. The enzyme is composed of four subunits of molecular weight of 55 000. The native molecule of final molecular weight of 220 000 aggregates in solution to multimers of molecular weight of 440 000 and higher. When subjected to isoelectric focusing the enzyme yields two components of equal distribution and pI-values of 5·15 and 5·25. The enzyme is thermolabile and is readily inactivated at temperatures above 3°C. The K_M value for adenosine is 5·15 $\mu mol\,l^{-1}$ and for S-adenosyl-L-homocysteine (SAH) 11 $\mu mol\,l^{-1}$. The temperature optimum of both SAH synthesis and hydrolysis is 37°C, the pH optimum of SAH hydrolysis is 8·0, of SAH synthesis 7·14. The enzyme is competitively inhibited by (*S*)-9-(2,3-dihydroxypropyl)adenine and inactivated by both enantiomers of eritadenine and 3-(adenin-9-yl)-2-hydroxypropionic acid.

Døskeland and Ueland[1] compared the characteristics of SAH hydrolases isolated under identical experimental conditions from beef liver, adrenal cortex and mouse liver and concluded that all these enzymes are tetramers made up most likely of two subunit types at a 1 : 1 ratio; they also postulated that the species and tissue differences which had been observed to exist in the kinetic and physico-chemical properties of the SAH hydrolases were due to methodical reasons.

The SAH hydrolase which was isolated from plant material significantly differs according to the data published so far, from the enzymes of animal origin: the native form of the SAH hydrolase from *Lupinus luteus* L. is allegedly a dimer[2,3]. Interest also deserves the finding that the plant enzyme is not inactivated by adenosine but, on the contrary, is somewhat stabilized by its presence[3]. Having regard to the above findings and also to the fact that open-chain adenosine analogs, virostatic agents which are strong inhibitors of animal SAH hydrolases, also show marked inhibitory effects on the growth of *Vicia faba*[4], we decided to isolate an additional plant SAH hydrolase. An explantate submersion culture of *Nicotiana tabacum* L. from the

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late logarithmic phase of growth was used to start with. Another aim of our study was to examine the inhibitory effects of the open-chain adenosine analogs and to verify the method of direct affinity purification using a specific ligand which had been employed to advantage for the isolation of animal SAH hydrolases[5,6].

In the first purification step the suspension of the *Nicotiana tabacum* tissue culture was vigorously cooled in liquid nitrogen and the cells powderized with Alumina A 305. A crude extract with SAH hydrolase activity was obtained by extraction of the material with 0·01 mol l^{-1} phosphate buffer at pH 7·4 containing 1 mmol l^{-1} dithiothreitol (DTT) and 0·07 g/ml of poly(N-vinylpyrrolidone). The crude extract was centrifuged and the proteins precipitated at 4°C in the supernatant by the addition of ammonium sulfate to 80% saturation. After the centrifugation the precipitate was dissolved in 0·01 mol l^{-1} phosphate buffer containing 1 mmol l^{-1} DTT and purified by affinity chromatography[5]. The enzyme preparation thus obtained showed a specific activity of 73 ncat mg^{-1} (4·4 EU mg^{-1}). A 40·7-fold purification was obtained with respect to the crude extract and the yield of the enzyme was 25% (Table I).

The purity of the preparation was verified by electrophoresis in SDS-polyacrylamide gel: one single component of molecular weight 55 000 ± 200 (Fig. 1)* was found. The molecular weight of the native enzyme was determined by electrophoresis in polyacrylamide gel of increasing density (4–20%). The electrophoresis was carried out in 10 mmol l^{-1} 2-mercaptoethanol and 1 μmol l^{-1} SAH; protein bands of molecular weight of 220 000 and 440 000 were detected. The former showed the main enzymatic activity after 15 h electrophoresis at 15°C (Fig. 2). When the addition of 2-mercaptoethanol was omitted considerable aggregation occurred during the electrophoresis and the enzyme penetrated into the gel to a negligible degree

TABLE I
Purification of SAH hydrolase from *Nicotiana tabacum* cells

Purification step[a]	Quantity of protein (mg)	Total activity[b] (ncat)	Specific activity (ncat mg^{-1})	Purification coefficient
Crude extract	37·5	66·85	1·78	—
Sulfate fraction	12·3	45·0	3·76	2·05
Affinity chromatography (effluent)[c]	0·238	17·17	72·68	40·7

[a] From 100 g of cells (wet weight); [b] the activity and specific activity were determined in direction of SAH synthesis; [c] after the separation of adenosine on Sephadex G-25 M.

* See insert on the page 1544.

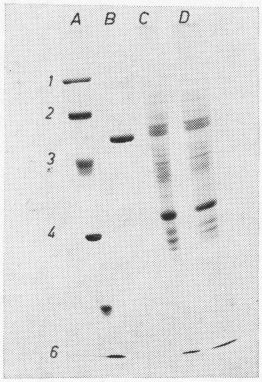


Fig. 1

SDS-polyacrylamide gel electrophoresis, *A* Electrophoresis calibration kit: *1* phosphorylase b, *2* bovine serum albumin, *3* ovalbumin, *4* carbonic anhydrase, *5* soybean trypsin inhibitor, *6* α-lactalbumin; *B* Pattern of SAH-hydrolase subunit after affinity chromatography; *C* crude extract after ammonium sulfate precipitation; *D* crude extract

only. The addition of SAH to the gel and to the electrode buffer resulted in a considerable suppression of the thermolability of the enzyme (see below) and therefore the activity could be determined in the gel even after 15 h electrophoresis at 15°C. Isoelectric focusing of tobacco SAH hydrolase demonstrated that the preparation obtained by us, which was homogeneous in terms of the molecular weight of both the subunit and the tetramer native form, yields two components of identical distribution and pI-values of 5·15 and 5·25 (Fig. 3).

The enzyme shows very steep temperature optima for both directions of the reaction catalyzed; in both cases the enzyme shows a temperature optimum of 37°C

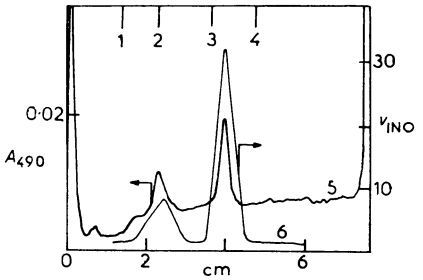


FIG. 2

Gradient gel electrophoresis. Scanning of SAH-hydrolase patterns after electrophoresis on 4—20% gradient polyacrylamide gel. HMW calibration standards *1* thyroglobulin, *2* ferritin, *3* catalase, *4* lactate dehydrogenase, *5* scanning of SAH-hydrolase patterns at 490 nm, *6* SAH-hydrolase activity in nmol l^{-1} h^{-1}

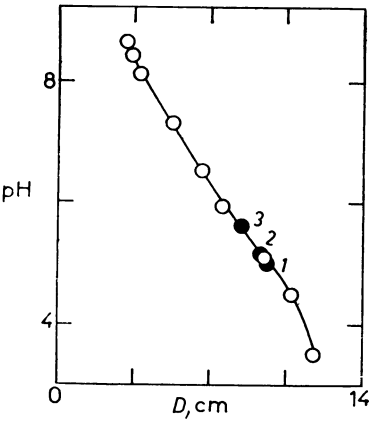


FIG. 3

Isoelectric focusing. *1*, *2* SAH-hydrolase from *Nicotiana tabacum*, *3* SAH-hydrolase from mouse leukemic cells L1210. Open circles IEF calibration kit, *D* distance from the cathode

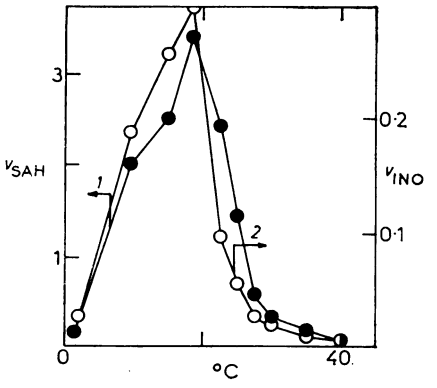


Fig. 4

Temperature optimum of SAH hydrolase; *1* SAH synthesis, *2* SAH hydrolysis. The reaction rate is expressed in μmol min^{-1} mg^{-1}

(Fig. 4). The effect of pH on the activity of the enzyme was also examined in terms of both directions of the reaction catalyzed. The pH-optimum of SAH synthesis significantly differs from that of SAH hydrolysis: the pH-optimum of SAH synthesis lies between 7·0 and 7·25, the maximum being 7·14, whereas the pH-optimum of SAH hydrolysis lies between 7·7 and 8·5 with a maximum at 8·0. The pH-optimum of the latter reaction is less sharp than that of the synthesis; a slow decrease of enzymatic activity manifests itself at higher pH-values (Fig. 5). The Michaelis constant (K_M) for adenosine was determined at constant L-homocysteine concentration (3·0 mmol . $.l^{-1}$) over the concentration range of 10–50 μmol l^{-1} adenosine and shows a value of 5·15 μmol l^{-1}. The K_M constant for SAH was determined at concentrations of 2–20 μmol l^{-1} and amounts to 11 μmol l^{-1}.

Neither SAH hydrolysis or synthesis by the plant enzyme are inhibited by the substrate, *i.e.* adenosine. The stability of the enzyme preparation was assayed in experiments with preincubation of the enzyme at a concentration of 0·8 μg ml^{-1} (SAH synthesis) and at 2·0 μg ml^{-1} (SAH hydrolysis) under the conditions shown in Table II. The enzyme was preincubated in all cases as a solution in 0·01 mol l^{-1} phosphate buffer, pH 7·4, containing 20% of glycerol (v/v). The residual enzymatic activity was determined after 5 min incubation at 37°C under standard conditions. The results are given in $\tau/2$ (a preincubation time during which the residual activity of the enzyme drops to one half).

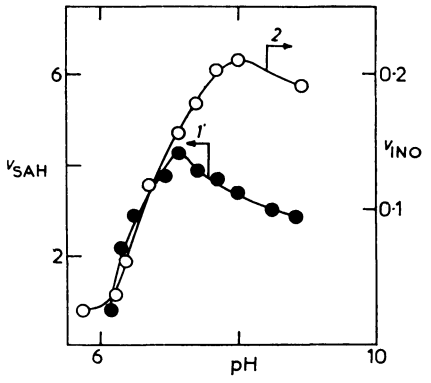


FIG. 5

pH-Optimum of SAH hydrolase; *1* SAH synthesis, *2* SAH hydrolysis. The reaction rate is expressed in μmol min^{-1} mg^{-1}

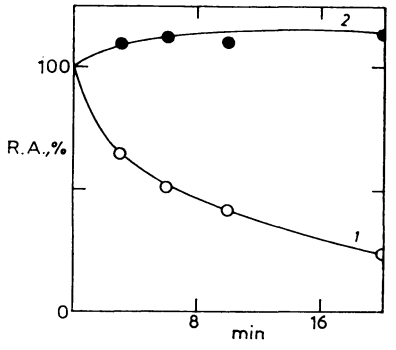


FIG. 6

Effect of SAH on inactivation of SAH hydrolase. The enzyme (0·95 μg ml^{-1}) in 20% glycerol, supplemented with serum albumin (50 μg ml^{-1}) was preincubated in phosphate buffer *1* and in the presence of 16·6 μmol l^{-1} of SAH 2; min time of preincubation at 37°C, R.A. residual enzyme activity

Isolated SAH hydrolase is an enzyme which is thermally very unstable and is readily inactivated at temperatures above 3°C, especially at low enzyme concentrations. The enzyme can be stabilized by the addition of a carrier protein (*e.g.*, by serum albumin) (Table II). A partial stabilization is brought about by the addition of adenosine (Table II) and a marked thermal stabilization can be achieved by the addition of SAH (Fig. 6). The stabilization by the presence of SAH manifests itself in Fig. 6 as an apparent activation of the enzyme: curve 2 represents the activity of the enzyme including SAH hydrolysis during the preincubation (without adenosine aminohydrolase) which, however, does not exceed 10% of residual activity after 20 min.

SAH hydrolase from *N. tabacum* was also used for testing the effects of several inhibitors whose action had been assayed before with the animal SAH hydrolases[6-8]. These inhibitors are analogs of the natural SAH hydrolase substrate, *i.e.* of adenosine, with a chemically modified sugar moiety of the molecule. The analogs assayed were (*S*)-9-(2,3-dihydroxypropyl)adenine (DHPA) (*I*) and related derivatives having the structure of ω-carboxylic acids (the enantiomers of eritadenine *II*, *III*, and 3-(adenin-9-yl)-2-hydroxypropanoic acids *IV*,*V*) which act as irreversible inhibitors of the enzyme. The most potent inhibitor of the above group similarly as in the case of the enzyme

TABLE II
Stability of SAH hydrolase during preincubation in 20% glycerol

Supplemented by	Synthesis			Hydrolysis		
	T, °C	pH	τ/2, s	*T*, °C	pH	τ/2, s
—	3	7·14	[a]	3	7·4	[a]
—	3	7·4	5 340	3	8·0	[b]
—	21	7·14	2 160	21	7·4	[c]
—	21	7·4	1 980	21	8·0	2 400
—	37	7·14	132	37	7·4	127
—	37	7·4	96	37	8·0	54
BSA[d]	37	7·4	114	37	7·4	408
Ado[e]	37	7·4	1 620	—	—	—
Ado[e], BSA[d]	37	7·4	1 800	—	—	—
L-HCys[f]	37	7·4	198	—	—	—
L-HCys[f], BSA[d]	37	7·4	276	—	—	—
—	45	7·14	54	45	7·4	48
—	45	7·4	24	45	8·0	36

[a] The residual activity of the enzyme after 120 min preincubation was >85%; [b] >55%; [c] >60%; [d] bovine serum albumin 50 μg ml^{-1}; [e] Ado adenosine 71 μmol l^{-1}; [f] L-HCys L-homocysteine 5 mmol l^{-1}.

from rat liver and of mouse leukemic cells[7,8], is D-eritadenine (*II*) which inhibits by 50% the hydrolysis of SAH in a concentration 2 500 times lower than the concentrations of the substrate. The inhibitory power of 3-(adenine-9-yl)-(2*R*)-hydroxypropanoic acid (*IV*) is close to that of D-eritadenine (Table III). The time course of the enzyme stability in the presence of irreversible inhibitors was tested only in the direction of the synthesis and the results were treated graphically according to Chiang and coworkers[9] (Fig. 7). The enantiomers of eritadenine and 3-(adenin-9-yl)-2--hydroxypropanoic acid cause inactivation which is of curvilinear character; therefore these analogs can be classified as belonging to group B (ref.[9]). DHPA is different from the above inhibitors. A 50% inhibition of SAH synthesis takes place

TABLE III
Inhibition of SAH hydrolase by aliphatic adenosine analogs

Inhibitor	Synthesis IC_{50}, nmol l^{-1} [a]	Hydrolysis IC_{50}, nmol l^{-1} [a]
I	28 000	1 500
II	26	1·5
III	720	3·7
IV	350	12·5
V	203	5·5

[a] The concentration of inhibitor which will bring about a 50% inhibition of the enzyme during 10 min incubation at 37°C, pH 7·4 (standard conditions).

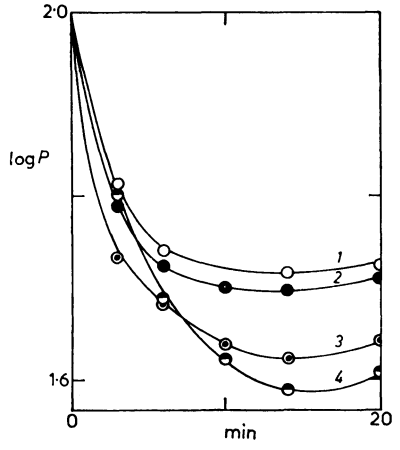
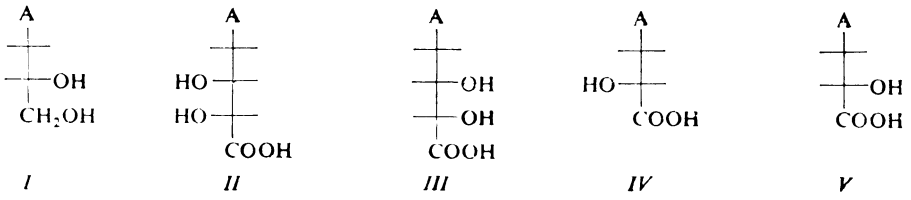


FIG. 7
Time profile of SAH hydrolase inactivation by reversible inhibitors. Effect of *1* 3-(adenin--9-yl)-(2*R*)-hydroxypropanoic acid (*IV*), *2* 3-(adenin-9-yl)-(2*S*)-hydroxypropanoic acid (*V*), *3* L-eritadenine (*III*), *4* D-eritadenine (*II*); min time of incubation, log *P* logarithmus of residual enzyme activity in direction of SAH synthesis

at a concentration roughly identical to the concentration of adenosine. This inhibition is reversible. The results are given in Table III. The inhibitory constant K_i for DHPA is 4·5 μmol l^{-1} as regards SAH synthesis and 0·95 μmol l^{-1} as regards SAH hydrolysis (K_i/K_M = 0·08).



I *II* *III* *IV* *V*

In formulae *I*—*V*, A adenin-9-yl residue.

It can be concluded that the characteristics of SAH hydrolase isolated from a *N. tabacum* tissue culture are very similar to those of the enzyme isolated from *L. lupinus* seeds[2,3]: identical or very similar values were found for the subunit size and the same holds for the K_M values for SAH and adenosine. The enzyme is not inhibited by the substrate and is not inactivated by either of its two substrates[2,3]. The molecular weight determination of the native enzyme from *N. tabacum* points to a tetramer arrangement of subunits in the molecule (unlike the SAH hydrolase isolated from *L. lupinus* seeds[2,3]). The enzyme shows a considerable aggregation ability, much higher than that of the enzymes isolated from animal sources[5,10,11]; this ability can be suppressed by the addition of 2-mercaptoethanol. We were not able to show in any of the experiments the ability of SAH hydrolase to form multimers composed of an odd subunit number as reported earlier[2]; we merely found multimers whose molecular weight is a multiple of 220 000 and whose enzyme activity is difficult to demonstrate after long-term equilibrium gel electrophoresis. The enzyme is rapidly inactivated at temperatures above 3°C. Since, however, an addition of SAH stabilizes the enzyme we were able to find enzymatic activity both in the component of molecular weight of 220 000 and in its dimer (Fig. 2).

The considerable thermal lability of SAH hydrolase from *N. tabacum* no doubt affects the resulting specific activity of the preparation isolated and thus also the purification coefficient. The analysis of the crude extract by SDS-polyacrylamide gel electrophoresis (Fig. 1) showed, however, that the apparently low purification coefficient (40·7) is in accordance with the relatively high distribution of the enzyme in the cytoplasm. The method of affinity chromatography enabled us a very rapid isolation of the homogeneous enzyme and thus verified its general applicability for the isolation of SAH hydrolases. In spite of this fact we cannot exclude the possibility of partial inactivation during the preparation.

EXPERIMENTAL

Materials. The suspension culture of *Nicotiana tabacum* L., KM-D clone was obtained by submersed cultivation according to Gamborg and coworkers[12]. The culture was harvested after 10 days during the late exponential phase of growth. (*S*)-9-(2,3-Dihydroxypropyl)adenine (*I*), D-eritadenine (*II*), L-eritadenine (*III*), 3-(adenin-9-yl)-(2*R*)-hydroxypropanoic acid (*IV*), and 3-(adenin-9-yl)-(2*S*)-hydroxypropanoic acid (*V*) were prepared according to [13,14]. The stock solutions of L-homocysteine and [^{14}C]-S-adenosyl-L-homocysteine were prepared according to[7]. [Adenine-U-^{14}C]-adenosine was a product of the Institute for Research, Production and Use of Radioisotopes (Prague, ČSSR). Adenosine aminohydrolase (E.C. 3.5.4.4) was from Boehringer (Mannheim, FRG), Coomassie Brilliant Blue R-250 and G-250 were from Fluka (Buchs, Switzerland), Bromophenol blue from Serva (FRG). Alumina A 305 was a product of Sigma (USA) and poly(vinylpyrrolidone) was from Calbiochem (USA). The Ampholin carrier ampholytes were purchased from LKB (Sweden) and the Electrophoresis calibration kit and IEF kit were from Pharmacia Fine Chemicals (Sweden).

Affinity chromatography. The purification of the enzyme was carried out on CH-Sepharose 4B modified by (*RS*)-9-[3(2)-(3-aminopropylamino)-2(3)-hydroxypropyl]-8-hydroxyadenine according to[5].

Enzyme activity assay. The synthetic and hydrolyzing activity of SAH hydrolase was measured under standard conditions[6-8] and the analysis of the reaction products was carried out by paper chromatography or by HPLC (ref.[8]). Sodium-potassium phosphate buffers ("phosphate buffer" in the following text) according to Sörensen were used in all experiments.

Electrophoresis in SDS-*gels.* The electrophoresis was carried out in a 15 × 12 cm slab 1·3 mm thick. A 4% sample gel, containing 0·125 mol l^{-1} Tris-HCl buffer at pH 6·8, a 10% running gel, containing 0·375 mol l^{-1} Tris-HCl buffer at pH 8·8, and Tris-glycine electrode buffer pH 8·3, containing 0·025 mol l^{-1} Tris and 0·192 mol l^{-1} glycine, were used. The buffer and the gels concontained 0·1% sodium dodecyl sulfate. The electrophoresis was allowed to proceed 3 h at 40 mA and a potential gradient of 150–270 V at room temperature. The sample was concentrated by precipitation in 10% trichloroacetic acid, washed with a solution of ethanol–ether (1 : 1, v/v), and dissolved in 0·01 mol l^{-1} disodium hydrogen phosphate at pH 7·0, containing 10% of glycerol 5% of 2-mercaptoethanol, 2·5% of sodium dodecyl sulfate, and 0·002% of Bromophenol blue. The solution was heated 3 min at 100°C and to the gel were applied 1·5 μg of the enzyme preparations and 2–5 μg of a Pharmacia Fine Chemicals standard for the determination of molecular weights. A sample of the crude extract was prepared by the same procedure and the quantity applied to the gel was 4–6 μg. The gel was stained overnight in 0·2% solution of Coomassie Brilliant Blue R-250 and destained in the system methanol–acetic acid–water (25 : 10 : 65). The apparatus for vertical electrophoresis and the solutions used were prepared according to Hames[15].

Electrophoresis in gel of increasing density. The molecular weight of the native enzyme was determined by electrophoresis in polyacrylamide gel of increasing density. The electrophoresis was run in 4–20% gel, containing Tris-borate buffer at pH 8·4 (0·09 mol l^{-1} Tris, 0·08 mol l^{-1} boric acid, 0·0025 mol l^{-1} Na_2EDTA) with 10 mmol l^{-1} 2-mercaptoethanol and 1 μmol l^{-1} SAH using model GE-4 Pharmacia Electrophoresis apparatus and the procedure recommended by the manufacturer[16]. Tris-borate buffer at pH 8·4, prepared according to the same procedure and also containing 10 mmol l^{-1} 2-mercaptoethanol and 1 μmol l^{-1} SAH, was used as the electrode buffer. The enzyme preparation (5·7 μg dissolved in 20% glycerol), concentrated by ultrafiltration, was applied to a 8 × 8 cm gel slab. The electrophoresis was allowed to proceed 15 h in the apparatus cooled by water at 150 V and 60–40 mA per one gel. The proteins were fixed

after completion of the run 30 min in 10% solution of sulfosalicylic acid, washed with the destainer and then stained in 0·2% solution of Coomassie brilliant blue R-250; they were destained 24 h in the system methanol–acetic acid–water. A set of proteins of defined molecular weights, supplied by Pharmacia Fine Chemicals, was used as a standard. A part of the gel was cut immediately after completion of the electrophoresis to 0·5 cm strips; the hydrolytic activity of SAH in the individual fractions was determined after 20 min incubation at 37°C. The distribution of the SAH hydrolase tetramer and octamer was determined by densitometric scanning of the stained strips at 490 nm in the gel.

Isoelectric focusing of SAH *hydrolase.* The isoelectric point of the enzyme preparation was determined by isoelectric focusing in 4% polyacrylamide gel in glass tubes and on a glass plate (26·5 × 20 cm; gel thickness 0·3 mm). The focusing was performed in 4% polyacrylamide gel containing 1% of Ampholine 3,5—7, a product of LKB (Sweden), in 0·5 × 12·5 cm tubes (6 h at 4°C, 2—0·27 mA per sample, potential gradient 340—400 V, prefocusing in the absence of sample for 20 min at 2 mA and a potential gradient of 320—380 V per tube). The anolyte was 0·01 mol l^{-1} L-aspartic acid and the catholyte 0·01 mol l^{-1} 2-aminoethanol. The quantity applied to one gel was 2·85 μg of the enzyme preparation in 20% glycerol, concentrated by ultrafiltration. The gels were fixed for 30 min in 10% trichloroacetic acid after the focusing, washed overnight in the system methanol–acetic acid–water, and stained by the same procedure as the gels after electrophoresis in polyacrylamide gel. To determine the pH-gradient, the gel was cut longitudinally and one half cut to 4 mm pieces. The latter were extracted overnight in 0·75 ml portions of 0·25 mol l^{-1} KCl; the pH of the individual samples was measured. Isoelectric focusing in an ultrathin layer was carried out in 4% polyacrylamide gel containing 1% of Ampholine 3—10 LKB on a 26·5 × 20 cm plate, 2 h at 1 000 V and 8°C; the medium was the same as that used for the tubes.

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