AFFINITY CHROMATOGRAPHY OF ARGINASE WITH CONTINUOUS DETECTION OF ENZYME USING UREASE MEMBRANE ELECTRODE

Lumír MACHOLÁN and Bohdana HLAVATÁ

Department of Biochemistry, J. E. Purkyně University, 611 37 Brno

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A rapid method is described of potentiometric measurement of liver arginase during its purification on a column of affinity adsorbent prepared by the derivatization of Spheron E-1 000. The detection of the enzyme in the flow-through system is effected by an ammonia electrode coated by the reaction layer of glutaraldehyde-crosslinked urease on a polyamide mesh. The method is time- and urease-saving and permits arginase to be obtained for analytical purposes purified 212 times from a liver extract in a 68% yield. By combining the membrane made of crosslinked arginase and urease on a polyamide mesh with a pNH_3 electrode we developed a two-enzyme sequence electrode sensitive to L-arginine (10^{-3}—10^{-4} mol l^{-1}) showing a response time of 1—3 min and a stability of about 3 weeks.

Enzyme electrodes as highly selective and sensitive biotechnological sensors have in many cases left research laboratories and started a progressive trend of reagent-free analyses in medical diagnostics, in food and fermentation technologies as well as in the monitoring of environment pollution[1]. Reports on the application of enzyme electrodes as analytical detectors in chromatographic separations, such as on the HPLC determination of acetylcholine and choline[2], are rather scarce.

In an effort to design a biosensor sensitive to the amino acid L-arginine we performed a one-step chromatographic purification of arginase from a thermally treated bovine liver extract with continuous monitoring of the arginase activity by a potentiometric urease electrode. Arginase adsorbed on the affinity adsorbent derived from Spheron E-1 000, a glycidylmethacrylate copolymer, was released in inactive state (after removal of the contaminant protein inpurities) by a neutral L-arginine solution. The column effluent was mixed with an alkaline buffer (enzyme activation) and the arginase content was subsequently monitored in a flow-through arrangement by a pNH_3 electrode coated with a layer of crosslinked urease. This nondestructive way of analysis permits an easy selection of fractions with the highest specific activity at the simultaneous monitoring of proteins by means of UV detection. It also saves time and urease necessary for the new discontinuous and lengthy photometric determination of arginase activity[3]. The procedures for the isolation of arginase from liver of cattle[4], sheep[4], horse[4,5], calf[6] and rat[7], which have been reported so far, involve up to seven purification steps.

EXPERIMENTAL

Material

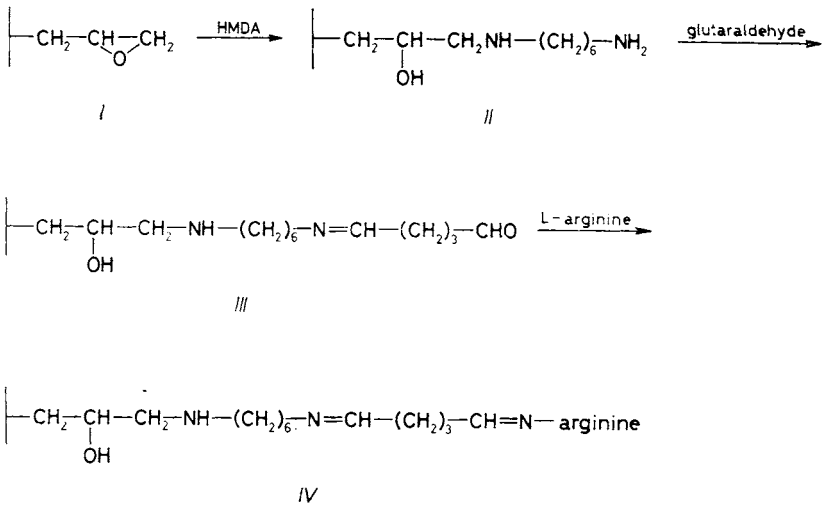
The glycidylmethacrylate copolymer Spheron E-1000 (particle size 0·2 mm), 1,6-hexamethylenediamine, and L-arginine hydrochloride were from Lachema, Brno. Jack bean urease (Sigma, type III, 334 nkat mg^{-1}), 25% aqueous glutaraldehyde solution (Fluka), bovine serum albumin (Mann Research Laboratories), and the remaining chemicals were not further purified. The arginase preparation used for calibration had a specific activity of 442 nkat mg^{-1} and was purified from bovine liver according to Bach and Killip[4] up to step E. As a support for the enzyme membranes served a commercial monofilament polyamide network (Silon) of fiber thickness 40 μm (25 mesh mm^{-2}).

Preparation of Liver Extract

Bovine liver (50 g) cut to pieces was homogenized 5 min under cooling with ice in 150 ml of 10 mM Tris-HCl buffer at pH 7·5 containing 50 mM-$MnCl_2$ and 0·1M-KCl. The homogenate was centrifuged (15 min at 15 000 *g*) and the pH of the supernatant was then adjusted to pH 7·5 by NaOH. The solution was heated to 55°C with stirring in a water bath (65°C), maintained at this temperature for 5 min and then cooled. The proteins precipitated were centrifuged off (10 min at 15 000 *g*), the supernatant was concentrated in an ultrafiltration apparatus (Amicon) and dialyzed against 10 mM Tris-HCl buffer at pH 7 not containing $MnCl_2$. The volume of the solution after concentration was 21·5 ml, the total arginase activity was 79·3 μkat (yield 79·8%) and the specific activity was 30 nkat mg^{-1} protein.

Preparation of Affinity Adsorbent

The first two steps were identical to those described in paper[8].



I *II* *III* *IV*

Spheron E-1000 (*I*, 10 g) was suspended in 50 ml of water and deaerated. After the addition of hexamethylenediamine (HMDA, 10 g) the suspension was heated 4 h in a water bath at 100°C. Product *II* was then filtered off by suction, washed thoroughly with distilled water, then with 50 ml of 6M urea, and then again with water. Wet derivative *II* was equilibrated 30 min in 100 ml of 0·1M phosphate at pH 7, filtered off by suction and again resuspended in the same volume of buffer. A 25% solution of glutaraldehyde (20 ml) was added and the suspension was shaken 4·5 h at room temperature. The brown-tinged product *III* was filtered off by suction, washed with buffer and suspended in 100 ml of 0·1M phosphate buffer at pH 7 containing 4 g of L-arginine hydrochloride. The suspension was shaken 3 h at room temperature and final product *IV* was washed thoroughly with distilled water, transferred to 10 mM Tris-HCl buffer at pH 7 and kept at 4°C. The quantity of immobilized arginine (about 10 μmol per 1 g of wet adsorbent) was estimated after 2 h hydrolysis in boiling 6M-HCl by paper chromatography.

Determination of Enzymatic Activity

The activity of arginase was assayed at 25°C by two different methods: either kinetically by the pNH_3 electrode coated by an urease layer in 0·1M Tris-HCl buffer at pH 8·7 containing 10 mM arginine (calibration with urea) and, for resons of comparison, by the photometric phenol--nitroprusside method[3] in 5 mM bicarbonate at pH 9·5 containing 10 mM arginine (Tris buffer cannot be used). After the activity of arginase had been determined by each method at both pH values the factor for the calculation of activity at pH 9·5 from the value determined at pH 8·7 was 1·64 and 1·67 for both methods, i.e. nearly identical to the value of 1·5 derived from the pH-curve[9]. Unless stated otherwise the activity indicated in this study is for practical reasons the activity of non-activated arginase (in the presence of the Mn^{2+}-salt the activity of the enzyme will increase 15 times).

Preparation of Enzyme Membranes

The solutions of 10% bovine serum albumin, 10% urease, and 2% glutaraldehyde were mixed together at a ratio of 3 : 8 : 4 and the mixture was spread over both sides of stretched polyamide mesh; it was dried in horizontal position at room temperature and disks 13 mm in diameter were cut afterwards with a sharp cork bore. These disks were employed for the detection of urea during the arginase activity assay. For the determination of L-arginine two enzyme membranes were prepared in a similar manner using 3 parts of 10% serum albumin, 8 parts of 10% urease, 3 parts of an arginase solution (133 nkat mg^{-1}) and 6 parts of 2% glutaraldehyde. The membranes were kept in dry state at 4°C.

Measuring Apparatus

The layout of the concept is shown in Fig. 1. The liver extract (1—1·5 ml) was placed onto a column (0·8 × 14 cm, K 9 Pharmacia) packed with 7 ml of affinity support *IV* in 10 mM Tris-HCl buffer at pH 7. After the column had been washed with the same buffer a neutral eluent solution (10 ml gradient of 0—1M-KCl and then 1M-KCl containing 2 mM arginine in 10 mM Tris-HCl buffer at pH 7) was pumped through the column at a rate of 0·33 ml min^{-1} using a peristaltic pump (Pharmacia P 3). The effluent was continuously mixed by means of a second pump (flow rate 0·33 ml min^{-1}) with 0·1M Tris-HCl buffer at pH 8·7 containing 1 mM EDTA. The mixture was passed through polyethylene tubing of 1·2 mm internal diameter via an injection valve (loop of 0·2 ml) and a mixing coil (length 50 cm) to a UV detector (VD ČSAV, Prague) and then to a thermostated cell (25°C) mounted on the front of the pNH_3 electrode (Orion

model 95—10) equipped with two membranes, i.e. the original membrane permeable for gaseous NH_3 and the urease membrane (oriented toward the flow of the solution). The enzyme electrode was connected through a precise digital pH-meter (Radelkis type OP-208) to a linear recorder operating at 7·5—100 mV over a chart scale of 250 mm. The whole apparatus was placed in an earthed Faraday cage. The buffers were deaerated and thermostated at 25°C to prevent the formation of bubbles.

RESULTS AND DISCUSSION

Detection of Arginase by Urease pNH_3 Electrode

The decisive role in the enzymatic-potentiometric assay of arginase activity is that of the reproducible preparation and the operational stability of the biosensor sensitive to one of the products of arginine cleavage, i.e. to urea.

$$\text{L-arginine} \xrightarrow{\text{arginase}} \text{L-ornithine} + \text{urea} \xrightarrow{\text{sensor membrane}} CO_2 + 2\,NH_3 \text{ (detection)}$$

In this study we employed, unlike other authors using electrodes for arginase assay with a gel[10] or liquid[11] urease layer, a better reproducible reaction layer. The letter consisted of polyamide mesh coated with a thin layer of urease crosslinked together with bovine serum albumin by glutaraldehyde. The electrode gave in the stationary arrangement of analyses a linear response over the concentration range of $1{\cdot}6 \cdot 10^{-5} - 1 \cdot 10^{-3}$ mol l^{-1} urea; the operational stability of the electrode was up to 14 days at room temperature.

Before the activity of arginase could be assayed in the liver extract the latter had to be freed of Mn^{2+}-ions strongly inhibiting urease in the sensor membrane. Under these conditions the activity of arginase decreased 15 times. This is of advantage for the monitoring of high activities during the purification of the enzyme on the affinity column. From Fig. 2 it can be seen that the urease pNH_3 electrode monitors

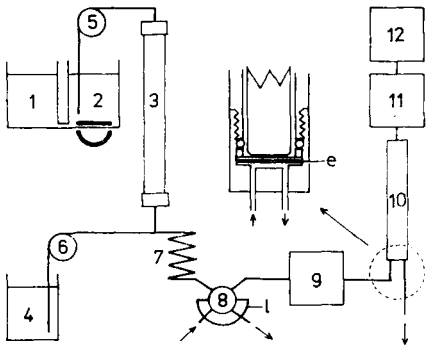


FIG. 1

Scheme of setup for separation and continuous recording of arginase. 1, 2 solutions for gradient elution; 3 microcolumn of affinity adsorbent; 4 alkaline buffer; 5, 6 peristaltic pumps; 7 mixing coil; 8 injection valve for urea and arginase standard; 9 UV detector; 10 urease pNH_3 electrode; 11 pH-meter; 12 recorder; e enzyme membrane; l sample loop

well the arginase reaction; the kinetics of urea formation assayed discontinuously both electrochemically and photometrically exhibits an identical time profile.

Monitoring of Arginase Eluted from Affinity Column

We have observed already in preliminary experiments that arginase is bound relatively strongly to affinity adsorbent IV. The enzyme is released very slowly by a neutral solution of 2–10 mM L-arginine solution and the elution time can be shortened significantly only by the addition of KCl. The enzyme is inactive at pH 7 (the pH-optimum lies at 9·5) and its detection would require a continuous mixing of the column effluent with a buffer at pH 8·7 (a higher pH value would increase the sensitivity of the pNH_3 electrode yet would simultaneously decrease significantly the activity of membrane-bound urease whose pH-optimum in the Tris buffer is 7·5). The addition of 1 mM EDTA to the buffer was made to protect urease against heavy metal ions. The length of the inlet tubing was chosen so that the mixture of arginase and the substrate might arrive in the electrode cell in one minute at a total flow rate of 0·66 ml min^{-1} for reasons of definition of the enzyme activity unit. When the elution gradient of 0–1 M-KCl was applied we met with another complication: the potential of the urease electrode varied with the increasing salt concentration

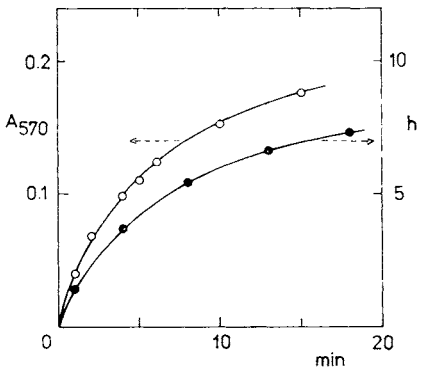


FIG. 2

Time course of arginase reaction as examined discontinuously by two independent methods. Samples were withdrawn from a buffered mixture of arginine and arginase (25°C) at time intervals and were analyzed photometrically (○) and by a flow-through urease electrode (●); *h* peak height in millivolts

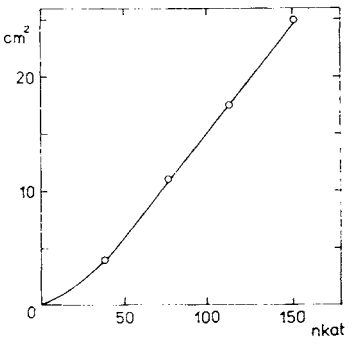


FIG. 3

Calibration of the flow-through system by arginase. Varying volumes of the standard arginase solution were applied to the column of derivative *IV*; the enzyme was eluted by a mixture of 1M-KCl and 2 mM arginine in 10 mM Tris-HCl buffer at pH 7 and continuously monitored at 25°C; enzyme activity at pH 8·7 is given on the abscissa and the corresponding peak area on the ordinate

towards positive values. The shift observed with 1M-KCl was up to 50 mV and NaCl showed the same effect. The sensitivity of the electrode to urea simultaneously decreased – in the case of 1M-KCl by one third. Luckily enough we observed that arginase is eluted from the affinity column only after contaminating proteins had been removed by the gradient; hence there were no problems with the calibration of the whole system by a known quantity of arginase standard in the presence of KCl in final concentration of 1 mol l^{-1}. In the flow-through arrangement the surface under the peaks was proportional to the activity or arginase applied to the column (Fig. 3).

Purification of Arginase by Column Chromatography

The scheme of the apparatus is shown in Fig. 1. The eluting solution containing 2 mM arginine and the activation buffer at pH 8·7 with 1 mM EDTA were pumped at the same rate (0·33 ml min^{-1}) by two peristaltic pumps through the mixing coil (length 50 cm) to the flow-through cell of the UV protein detector and then to the pNH_3 electrode equipped with a flow-through adapter. A typical elution diagram of the purification of thermally treated bovine liver extract is shown in Fig. 4. It can be seen that the column containing 7 ml of the affinity adsorbent can bind almost all arginase from 1·5 ml of extract corresponding to 3·5 g of liver tissue. The contaminating proteins are eluted by the Tris buffer and the rest by the buffered KCl gradient.

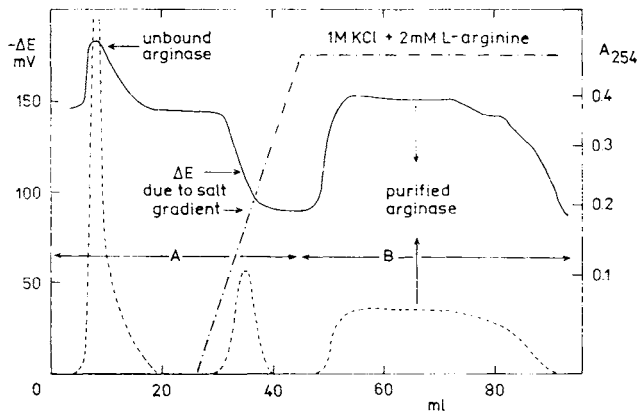


FIG. 4

Chromatographic separation of arginase from 1·5 ml of liver extract at pH 7 and 25°C. The UV absorbance is shown by a dashed line. For detection of arginase the effluent of given volume was mixed (1 : 1) with 0·1M Tris-HCl buffer at pH 8·7 without arginine (A) and with the same buffer containing 2 mM arginine (B)

Arginase can be released only by the substrate in 1M-KCl as the last component in a broad peak. When the pH of the eluent is changed by one half unit to both sides of the neutral point the total elution profile as well as the parameters of eluted arginase remain similar (Table I). The activity of the enzyme calculated from the area of the elution band obtained when the whole separation system was calibrated by a known quantity of arginase is in good agreement with the results of the photometric assay of total activity. After the activity was recalculated for pH 9·5 and 37°C (by multiplying the value by factors 1·65 and 2·04) the specific activity of the purified enzyme was 10·7 μkat/mg protein; this value is about twice as high as that of commercial preparation of Sigma. The arginase solution can be kept frozen at −20°C for several years.

The purification procedure described is very efficient since it involves three operations only: the homogenization of liver, thermal denaturation of the extract and

TABLE I

Affinity chromatography of arginase at 25°C and various pH. A volume of 1·5 ml of crude liver extract containing 184 mg of protein and 5·53 μkat of arginase was always applied to the affinity column (7 ml). The activity corresponds to the enzyme activated in 50 mM-$MnCl_2$

pH during elution	Active fraction ml	Total activity μkat	Total protein mg	Specific activity $\mu kat/mg^{-1}$	Degree of purification	Yield %
6·5	87	4·34	1·44	3·02	100	79
7·0	87	4·67	1·46	3·19	106	85
7·5	112	4·52	1·50	3·00	100	82

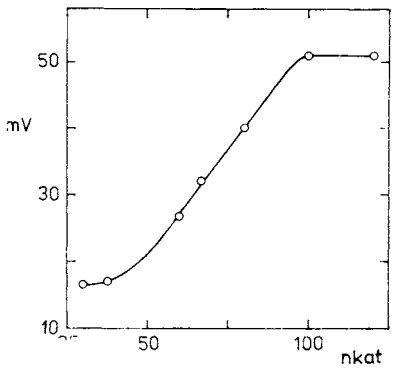


FIG. 5

Change of Nernst factor (mV per concentration decade) of two-enzyme electrode for arginine as function of quantity of arginase (nkat) in membrane. Arginase was cross-linked with 534 nkat of urease and 0·6 mg of bovine serum albumin by 12 μl of 2% glutaraldehyde over 133 mm^2 of polyamide mesh. The assay was carried out at 25°C and pH 8·7

affinity chromatography of the supernatant. Since about 20% of arginase activity is lost already during the thermal denaturation of the liver extract (the specific activity increases, however, to double its value) then, provided that the loss during chromatography is 15%, a total yield of 68% is obtained and the degree of purification of the final preparation is 212-fold with respect to the original extract. A disadvantage of the method is the small adsorption capacity of the column bed and especially the slow elution of the enzyme. If an extract of 100 g of liver were to be applied a column containing about 300 ml of adsorbent would be needed and the total volume of enzyme eluted would be about 3 liters. We have been able to rapidly dialyze such a large volume of effluent and simultaneously to concentrate it to 50 ml in a hemodialyzer (Chiraplat, Chirana, Stará Turá) in about 10 h. The affinity adsorbent *IV* can be used repeatedly after it has been washed with the starting buffer.

Two-Enzyme Electrode for L-*Arginine*

The principle of the function of the electrode for the determination of L-arginine, which has been designed by us, is the same as that of other types described, such as the electrode with a liquid layer of arginase and urease[12] or with a liver slice in combination with urease[13], i.e. arginine is sequentially degraded in the sensor membrane to electrochemically detectable ammonia. Our version uses crosslinked arginase and urease on a fine polyamide mesh. The urease content is the same as in the electrode for urea (534 nkat per membrane of an area of 133 mm^2) and the arginase content was optimized (100–120 nkat per membrane). The dependence of Nernst factor on arginase content in the active electrode layer is shown in Fig. 5. The highest value measured was 51 mV per concentration decade. The electrode showed in 0·1M Tris-HCl buffer at pH 8·7 and containing 1 mM EDTA linear calibration graphs over the range 0·1–1 mmol of arginine per liter and a response time of 1–3 min. The sensitivity of the electrode increased with the increasing pH (9·0–9·5) only slightly and the Nernst factor remained nearly unchanged (48 and 49 mV per concentration decade).

When the two-enzyme membrane was kept in 0·02% sodium azide at room temperature the slope of the calibration line decreased by 6·5 mV only in a week and by 12·5 mV in two weeks. When the dry membrane was stored at 4°C the slope also dropped by 12·5 mV in 14 days and by 14 mV in 3 weeks. The sensitivity decrease is no doubt a result of the limited stability of the enzymes immobilized: when ammonium chloride was tested the slope dropped from 57 mV to 55 mV per concentration decade during the same period. It should be noted for reasons of comparison that the pNH_3 electrode with a liquid layer of a urease and arginase mixture[12] showed a lifetime of 4 days only at room temperature and a response time of 5min over a linear $3 . 10^{-5}$–$3 . 10^{-3}$M arginine range.

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