Determination of polyamine oxidase activities in human tissues

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Summary. A very simple fluorometric assay for polyamine oxidase (PAO) in tissues, with N^1-monoacetylspermine as substrate, is described. The PAO was present in all human organs tested; it was highest in the liver, followed by the testis, kidney, spleen and small intestine.

Polyamine oxidase (PAO) was discovered in rat liver by Hölttä[1] in 1977. This enzyme is flavin-containing and is able to transform spermine into spermidine, and spermidine into putrescine in the presence of benzaldehyde. However, recently, Seiler's group[2,3] has demonstrated that N^1-monoacetylspermidine, N^1-monoacetylspermine and N^1,N^{12}-diacetylspermine are much better substrates for PAO than the non-acetylated polyamines; they may be the natural substrates for this enzyme, since various acetylpolyamines have been identified in human urine[4]. PAO attacks an imino group inside the chain of an acetylpolyamine and thus splits it to form a shorter polyamine, 3-acetamidopropanal and hydrogen peroxide[2,3].

In the present study, we have devised a simple fluorometric assay for PAO with N^1-monoacetylspermine as substrate and determined its activities in various human organs.

Materials and methods. Human organs were obtained from 4 male and 3 female cadavers of 23–69 years of age, victims of cardiac failure, pneumonia, hepatic cirrhosis, bronchial asthma, exsanguination and drowning, at forensic autopsies. The lapse of time after death was 3.5–22.5 h. The liver and spleen from the victim of hepatic cirrhosis and the lung from the one with bronchial asthma were not used.

The pieces of human organs were homogenized in 15 vol. of water with a Polytron homogenizer. The crude homogenate was used as an enzyme source. N^1-Monoacetylspermine and other acetylpolyamines, synthesized according to Tabor et al.[5], were kindly donated by N. Seiler, Centre de Recherche Merrell International, Strasbourg, France.

The present assay method for PAO is based in principle on the method of Guilbault et al.[6] and Snyder and Hendley[7], in which hydrogen peroxide formed in the oxidase reaction is measured fluorometrically by converting homovanillic acid to a highly fluorescent compound in the presence of peroxidase.

Results. The effect of pH on the PAO reaction was examined with the human liver enzyme and with N^1-monoacetylspermine as substrate; 0.5 M potassium phosphate and sodium borate buffer solutions covering pH 6.5–10 were used. The activity markedly increased up to pH 9.0 and remained at the same level up to pH 10.0. Various amines (final 0.1 mM), such as N^1-monoacetylspermine, N^1,N^{12}-diacetylspermine, N^1-monoacetylspermidine, N^8-monoacetylspermidine, monoacetylcadaverine, monoacetylputrescine, spermine, spermidine, cadaverine and putrescine, were tested for their oxidation by PAO in human liver in the presence of 0.1 mM pargyline, a monoamine oxidase (MAO) inhibitor, and 1.0 mM semicarbazide, a diamine oxidase (DAO) inhibitor. N^1-Monoacetylspermine showed the highest activity, and N^1,N^{12}-diacetylspermine and N^1-monoacetylspermidine showed 71.7 and 47.5% of it, respectively; all other substrates showed either no or negligible activities.

On the basis of the above data, the following procedure was adopted as a standard assay for PAO in human tissues. The assay mixture (total 0.6 ml) contained 0.1 ml of 0.5 M sodium borate buffer (pH 9.0), 0.1 ml of horseradish peroxidase solution (0.4 mg/ml), 0.1 ml of homovanillic acid solution (1.0 mg/ml, final concentration 0.92 mM), 0.05 ml of semicarbazide-HCl solution (1.3 mg/ml, final concentration 1.0 mM), 0.05 ml of pargyline-HCl solution (0.23 mg/ml, final concentration 0.1 mM), 0.1 ml of the homogenate (5.7–8.0 mg of wet tissue) and 0.1 ml of N^1-monoacetylspermine solution (0.42 mg/ml as a 3HCl salt, final 0.2 mM). Prior to the final addition of homovanillic acid and the substrate N^1-monoacetylspermine, the mixture was preincubated at 37 °C for 20 min. After incubation at 37 °C for 30 min, the enzyme reaction was stopped by adding 2.0 ml of 0.1 N NaOH solution, since the addition of the alkaline solution to the mixture results in a 3- to 4-fold increase in sensitivity[8]. Then, the fluorescence intensity was measured with excitation at 323 nm and emission at 426 nm (uncorrected). As a blank test, the assay mixture without substrate was incubated and the substrate was mixed after inactivation of the enzyme with the NaOH solution. Internal standards were taken to correct for quenching due to crude enzyme preparations, for each measurement, by adding 1.1–2.2 nmol of hydrogen peroxide in place of the substrate solution.

Under these conditions, the assays were linear during incubation for at least 60 min and also with respect to enzyme concentrations. The detection limit of the assay was 0.1 nmol per tube.

The apparent K_m value of the human liver enzyme for N^1-monoacetylspermine was less than 1.5 μM. The value of the rat liver enzyme for the same substrate was reported to be 0.6 μM[3]. Thus, the enzyme seems to be well saturated by the substrate under our assay conditions (substrate, 0.2 mM).

The table shows PAO activities in crude homogenates of various human organs. PAO was detected in all organs tested, although its activity was much lower than that in rats[2]. The highest activity was found in the liver, followed by the testis, kidney, spleen and small intestine.

Discussion. In the present communication, we have presented a detailed procedure of an extremely simple fluorometric assay for measurement of PAO in crude tissue homogenates. Hölttä[1] employed radioactive spermine and spermidine as substrates for PAO assays and separated their products by paper electrophoresis. Seiler et al.[2] measured PAO activities by isolating dansyl derivatives of the reaction products by TLC followed by fluorometric analyses. The present assay is much simpler and quicker than the previously-reported methods[1,2]. It does not need chromatographic separations and allows the assays of more than 50 samples within 3 h.

Seiler et al.[2] recommended N^1,N^{12}-diacetylspermine as substrate for PAO. However, PAO first splits N^1,N^{12}-diacetylspermine to form N^1-monoacetylspermidine and 3-acetamido-

PAO activities in various human organs*

Organ	PAO activity** (nmol/g wet weight/30 min)
Liver	398 ± 83 (6)***
Testis	339 ± 80 (4)
Kidney	263 ± 34 (7)
Spleen	140 ± 48 (6)
Small intestine	41.4 ± 11.7 (7)
Heart	37.6 ± 4.9 (7)
Brain	34.5 ± 5.1 (7)
Lung	31.7 ± 6.2 (6)
Pancreas	15.9 ± 6.9 (7)

* The final concentration of the substrate N^1-monoacetylspermine was 0.2 mM. ** Mean values ± SE are given. *** The number of samples is in parentheses.

propanal; the N^1-monoacetylspermidine can further react with PAO to form putrescine and the second molecule of 3-acetamidopropanal[3]. This complicated two-step reaction seems undesirable for PAO assays. The PAO activity with N^1-monoacetylspermine was higher than that with N^1,N^{12}-diacetylspermine. These are reasons why we have adopted N^1-monoacetylspermine rather than N^1,N^{12}-diacetylspermine as substrate for PAO in the present assay system.

We added pargyline, a potent MAO inhibitor, and semicarbazide, a DAO inhibitor, to the mixture, to ensure specific assays for PAO. These inhibitors had almost no effect on PAO activity at the concentrations used. We have reported that N^8-monoacetylspermidine, monoacetylcadaverine and monoacetylputrescine can be substrates for rat liver mitochondrial MAO although their affinity for this enzyme is very low, but N^1-monoacetylspermine, N^1,N^{12}-diacetylspermine and N^1-monoacetylspermidine are absolutely not oxidized by rat liver MAO[9]. All monoacetylpolyamines and diamines are able to be oxidized by hog kidney DAO although their K_m values are more than 5 mM[9]. Therefore, both inhibitors might be omissible in the assays of PAO in most mammalian tissues with 0.2 mM N^1-monoacetylspermine as substrate.

This is the first report to describe the occurrence of PAO in human tissues. Seiler et al.[2] measured PAO activities in rat tissues and reported that the highest activity was found in the pancreas, followed by the liver, spleen and kidney; the activity in the testis was much lower than in the kidney. In human tissues, however, the highest activity was observed in the liver and the activity in the pancreas was lowest (table), although the latter might be partly due to the post-mortem degeneration of PAO by proteolysis. We can recommend human liver as a good PAO source for purification studies. The present assay method seems very useful for enzymological studies on PAO because of its simplicity and sensitivity.

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0014-4754/84/080838-02$1.50 + 0.20/0

Stabilization of the egg-shell of a monogenean *Dionchus remorae*

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Summary. The phenolase of *Dionchus remorae* exists as proenzyme and is not substrate specific oxidising mono, di and polyphenols. The egg-shell is stabilized by quinone tanning.

The egg-shell of helminths is usually stabilized by quinone tanning[2]. Recent studies have shown that the egg-shell of monogeneans *Pseudomicrocotyle* sp., and *Pricea multae* are stabilized by dityrosine linkage and S-S bonding and that quinone tanning is absent in these species[3]. The present investigation reports the results obtained on the egg-shell stabilization of *Dionchus remorae.*

Materials and methods. The eggs of *D. remorae* were detached from the terminal ends of the gill filaments of *Scomberoides tol* and washed repeatedly in distilled water to free them of mucus. A 5 mg sample of eggs was hydrolyzed in 5 ml of 6 N HCl in a sealed test tube in an oven for 12 h at 105 °C. Excess HCl was evaporated in vacuo and by heating in a water bath at 65 °C. The sample was diluted with a few drops of water and further evaporated. The resultant amino acids were dissolved in 10% (v/v) isopropanol and spotted on Whatman No. 1 chromatography paper. Any acid remaining in the sample was neutralised by placing the paper over ammonia vapor. 2-way descending chromatograms were run using solvents butanol:acetic acid:water (12:3:5) for the first run (18 h) and phenol:water:ammonium hydroxide (80:40:1) in the second run. The amino acids were located using 0.2% ninhydrin in acetone. Phenols present in acid hydrolysate were visualized by their reaction with diazotized sulphanilic acid on a single dimensional chromatogram run with butanol:acetic acid:water[4]. The solubility of the eggs was tested by incubating samples in 1 N hydrochloric acid, 1 M sodium hydroxide or 0.75 M sodium hypochlorite for 48 h.

The phenolase in *D. remorae* vitellaria, i.e. vitelline glands situated in the lateral parts of the fluke and believed to contribute shell and yolk materials for egg formation, was detected by the following method[5]. Fresh, unfixed specimens, specimens treated with 0.2% sodium oleate for 15 min (prepared in phosphate buffer pH 6.8) and fresh specimens injured at the vitellaria were incubated separately with 0.1% aqueous solution of the substrates L-tyrosine, tyramine, DL-Dopa, dopamine, protocatechuic acid, catechol, epinephrine (adrenaline), pyrogallol, resorcinol or hydroquinone. Control experiments were carried out by incubating sodium oleate treated and injured specimens with phenylthiourea or diethyldithiocarbamate for 15 min prior to incubation with substrates. The enzyme activity was determined based on the brown color development in the experiments and absence of colour development in the controls.

Results and discussion. The solubility tests revealed that the egg-shell protein was insoluble in dilute acid and alkaline solutions, suggesting that covalent bonds were involved in stabilization. On prolonged treatment (36 h) with the detanning agent, sodium hypochlorite, the egg-shell was rendered soluble, indicating the possible presence of quinone bonds[6]. Chromatograms of the acid hydrolysate of *D. remorae* eggs had 19 ninhydrin-positive spots. The relative color intensities of the identified amino acids were in the order of: glutamic acid > aspartic acid > lysine > arginine > glycine > cystine > isoleucine > leucine > alanine > threonine > valine > serine > taurine > phenylalanine > histidine > proline > tyrosine and 2 unidentified spots (fig.).

The single dimensional chromatograms with diazotized sulpha-