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Note

Sensitive assay for diamine oxidase activity using high-performance liquid chromatography

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Diamine oxidases [diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6] (DAO) catalyse the oxidation by molecular oxygen of various diamines, such as putrescine and cadaverine, to the corresponding amino-aldehydes, hydrogen peroxide and ammonia. The aminoaldehydes are in equilibrium with their cyclic condensation products, Δ^1-pyrroline and Δ^1-piperideine, for putrescine and cadaverine, respectively (Fig. 1).

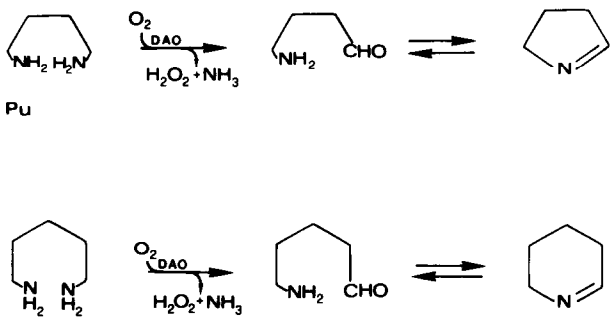

Fig. 1. Scheme of DAO-catalysed oxidation of putrescine (Pu) and cadaverine (Ca).

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Since DAO activity has been considered a useful parameter in various clinical studies [1, 2], many different assays for DAO activity have been proposed. Methods using radioactive substrate [3, 4] achieve maximum sensitivity, but often only simple instrumentation is available in analytical laboratories and alternative procedures are demanded. In this connection, the potentials of high-performance liquid chromatography (HPLC) have been recently exploited by Pietta et al. [5]. Their method estimates hydrogen peroxide using coupled peroxidase-catalysed oxidation of a fluorogenic compound.

Because of the criticism by Neufeld and Chayen [6] of coupled peroxidase assays we avoided this approach. It was our aim to reach the sensitivity of the method of Pietta et al. [5] by direct evaluation of a stable derivative of the reaction product. For this purpose the classical method of Holmsted et al. [7] was followed. This method is based on the treatment of Δ^1-pyrroline with *o*-aminobenzaldehyde (OAB) to give a condensation product (2,3-trimethylene-1,2-dihydroquinazolinium ion) which is spectrophotometrically analysed. Sakamoto and Samejina [8] modified this procedure by converting the condensation product to the 2,3-trimethylene-4-quinazolone (triMQ, Fig. 2) which can easily be estimated by gas chromatography with a higher sensitivity than that attained by spectrophotometry.

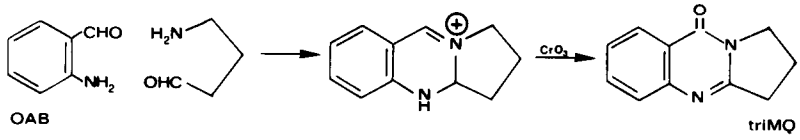
By exploiting the still higher sensitivity of HPLC we expected to obtain at the same time a more rapid method than that described by Sakamoto and Samejina [8]. In order to demonstrate the suitability of the described procedure, enzymatic activity in pea seedling extracts and bovine plasma samples was measured.

EXPERIMENTAL

Materials

Putrescine dihydrochloride and cadaverine dihydrochloride as well as ethyl acetate for HPLC were purchased from E. Merck (Darmstadt, F.R.G.). OAB was prepared by reducing *o*-nitrobenzaldehyde [9]. A solution of Δ^1-pyrroline was obtained by treating γ-aminobutyraldhyde diethyl acetal (Janssen, Beerse, Belgium) with 50 m*M* hydrochloric acid.

DAO from pea seedlings was prepared according to the method of Hill [10] up to Step 4, giving an enzymatic preparation with a protein content of 1.87



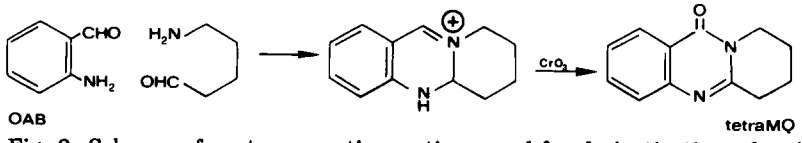


Fig. 2. Scheme of post-enzymatic reactions used for derivatization of aminoaldehydes which are produced by reaction of DAO with putrescine and cadaverine.

mg/ml. Bovine plasma samples were obtained in heparinized tubes by routine venous puncture from a normal healthy adult animal and stored frozen until analysis.

Pure triMQ was obtained on a preparative scale from Δ^1-pyrroline according to the procedure described below. 2,3-Tetramethylene-4-quinazolone (tetraMQ, Fig. 2) was obtained in the same manner from Δ^1-piperideine. This was produced by reaction of pea seedling DAO with cadaverine.

Chromatographic conditions

The HPLC unit consisted of a Twincle pump, an injector and a Uvidec 100 III ultraviolet detector (Jasco, Japan). Analyses were performed using a LiChrosorb Si 100 column (250 × 4.6 mm, particle size 7 μm) (Merck). Ethyl acetate was used as an eluent at a flow-rate of 2 ml/min. Absorbance was measured at 268 nm.

Post-enzymatic reaction conditions

In a set of screw-capped test tubes, samples containing 0.1 ml of 10% trichloroacetic acid and 20 nmol of Δ^1-pyrroline in 1 ml of aqueous solution were treated with 0.1 ml of 10 m*M* OAB for different times at various temperatures (room temperature, 50°C and 80°C). Then 0.5 ml of 1 *M* CrO_3 in 2 *M* sulphuric acid was added and the mixtures were kept for different times at the temperatures mentioned above. The solutions were made alkaline with 5 *M* sodium hydroxide and extracted with 3 ml of diethyl ether containing 20 nmol of tetraMQ, which served as internal standard. The organic phase was removed and a second extraction with 3 ml of diethyl ether was performed. The combined organic phases were evaporated to dryness; the residue was redissolved in 50 μl of ethyl acetate and 5-μl portions were analysed by HPLC. By comparing ratios between peak heights of triMQ derived from Δ^1-pyrroline and tetraMQ, the yield of the reaction was evaluated. Maximum yields were obtained either at room temperature after 2-h treatment with OAB and 4-h treatment with CrO_3 or by two treatments of 15 min at 80°C. These latter conditions were used in the subsequent experiments.

Calibration curve

In 1 ml of 25 m*M* phosphate buffer pH 6.5, amounts of cadaverine varying between 1 and 20 nmol were enzymatically converted to Δ^1-piperideine by addition of pea seedling DAO and OAB in a large excess and warming the solution at 37°C for 20 min. The reaction was stopped by the addition of 10% trichloroacetic acid. Then 10 nmol of Δ^1-pyrroline were added as internal standard and the mixtures were subjected to the procedure described above except that tetraMQ was not added before the extraction step. TetraMQ and triMQ obtained from cadaverine by enzymatic oxidation and from Δ^1-pyrroline, respectively, were analysed by HPLC and the ratios between their peak heights were calculated and plotted against the amounts of cadaverine.

DAO essay

Various aliquots (2—5 μl) of the enzyme preparation from pea seedlings or different volumes of bovine plasma (0.2—0.5 ml) were diluted to 1 ml with

phosphate buffer (pH 6.5 for pea seedling extracts, pH 8.0 for plasma). After the addition of 1 μmol of cadaverine and 1 μmol of OAB the mixtures were kept for 10 min at 37°C. The reaction was stopped with 10% trichloroacetic acid and the mixtures were treated as described above. DAO activity was calculated from the amount of tetraMQ formed by enzymatic oxidation of cadaverine:

$$\text{Activity (mU/ml)} = \frac{\text{nmoles tetraMQ}}{\text{10 (min)} \times \text{sample volume (ml)}}$$

One U is defined as the amount of enzyme which catalyses the oxidation of 1 μmol of substrate per min under the described conditions.

RESULTS

In Fig. 3 the HPLC profile of an enzymatic assay is shown. Under the conditions described the retention times of tetraMQ and triMQ were 3.9 min and 6.8 min, respectively.

A linear relationship between the ratios of the peak heights (R_h) of tetraMQ and triMQ and the amount of oxidized substrate was found in the range 1—20 nmol according to the equation

$R_h = 0.532 \times$ oxidized cadaverine (nmol) + 0.091 ($r = 0.997$)

By this method tetraMQ could be determined in amounts at least as low as 0.2 nmol, and therefore 0.02 mU of DAO could be detected. The reproducibility of the method was determined by repeated analyses of the same DAO preparation from pea seedlings. Enzyme activity and reproducibility were 21.5 ± 0.82 mU/ml (mean ± S.D., $n = 5$).

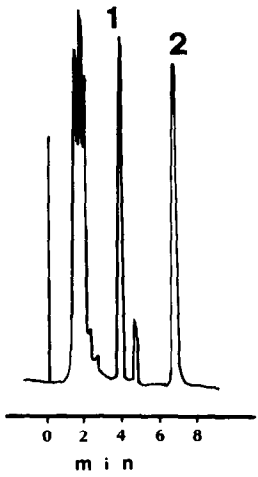


Fig. 3. HPLC profile of a sample assayed for DAO activity. Peaks: 1 = tetraMQ; 2 = triMQ.

Five bovine plasma samples obtained from the same animal on different days during two weeks were tested. The DAO activity was found to be between 0.92 and 1.32 mU/ml with a mean of 1.14 mU/ml.

DISCUSSION

The reactions shown in Fig. 2, which were described by Sakamoto and Samejina [8], were shown to be a reliable and simple basis for a method to trap short-chain aliphatic aminoaldehydes and allow the determination of the DAO-catalysed oxidation product of cadaverine and putrescine. By increasing the temperature, the previously reported reaction times can be decreased. Because of the stability of triMQ and tetraMQ, samples can be stored for several hours at room temperature without loss.

Cadaverine was preferred to putrescine as substrate because of the commercial availability of γ-aminobutyraldehyde diethyl acetal. Thus Δ^1-pyrroline could be used as internal standard.

The extraction of triMQ and tetraMQ from aqueous solution in an organic phase eliminates possible interferences deriving from biological material. Moreover, the convenient evaporation of organic solvents allows highly concentrated samples to be obtained, thus increasing the detection sensitivity. The separation of triMQ and tetraMQ by HPLC on silica is very effective and the short retention times are suitable for routine analysis.

The present method does not reach the sensitivity of the radiochemical method, which is still the procedure most used in putrescine metabolism studies [11]. We can state, however, that, because of its high reliability, the described method appears to be a useful alternative procedure for DAO activity assay in plant extracts and animal plasma samples. Additional experiments are required to apply this method to tissue homogenates.

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