PAPER CHROMATOGRAPHIC DETECTION AND ESTIMATION OF L-AMINO ACID OXIDASE OF SNAKE VENOM

R. W. P. MASTER*

The Haffkine Institute, Parel, Bombay (India)

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The L-amino acid oxidase activity in snake venoms is usually estimated by any of the following methods: (a) manometric method where oxygen consumption is measured, (b) estimation of ammonia produced, (c) colorimetric estimation of keto acid produced by the L-amino acid oxidase in presence of catalase and (d) the estimation of hydrogen peroxide produced in absence of catalase[1]. Recently, WEISSBACH and coworkers[2] have described a spectrophotometric procedure for the determination of L-amino acid oxidase in snake venoms. BOMAN AND KALETTA[3] have used a filter paper spot test, where remaining L-leucine is determined after oxidation with the snake venom enzyme, by the ninhydrin reaction.

While working on the enzyme systems in the venoms of Indian snakes, a paper chromatographic method was developed to detect and estimate L-amino acid oxidase. This method can also be used for the detection and determination of D-amino acid oxidase and histidase.

In the absence of catalase, L-amino acid oxidase acts on L-amino acids according to the following scheme[1].

$$RCH(NH_2)COOH + O_2 + H_2O \rightarrow RCOCOOH + NH_3 + H_2O_2$$

$$RCOCOOH + H_2O_2 \rightarrow RCOOH + CO_2 + H_2O.$$

With L-histidine as the substrate, the product of reaction would be imidazole acetic acid according to the reaction given above. The imidazole acetic acid formed is detected and estimated over the range 5–40 μg using diazotized sulphanilic acid as the spray reagent.

PROCEDURE AND RESULTS

Materials and methods

Venoms. The lyophilized venoms of cobra and Russell's viper collected at the Haffkine Institute were used. L-Histidine hydrochloride was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, urocanic acid was kindly supplied by Dr. MARIAN W. KIES of N.I.H. (U.S.A.) and imidazole acetic acid was a gift from Dr. HERBERT TABOR, (N.I.H.) U.S.A. A 1% solution of the lyophilized venom in distilled water was prepared. Aqueous solutions (2 mg/ml) of L-histidine hydrochloride,

* Present address: Division of Enzymology, Institute of Medical Research, The Chicago Medical School.

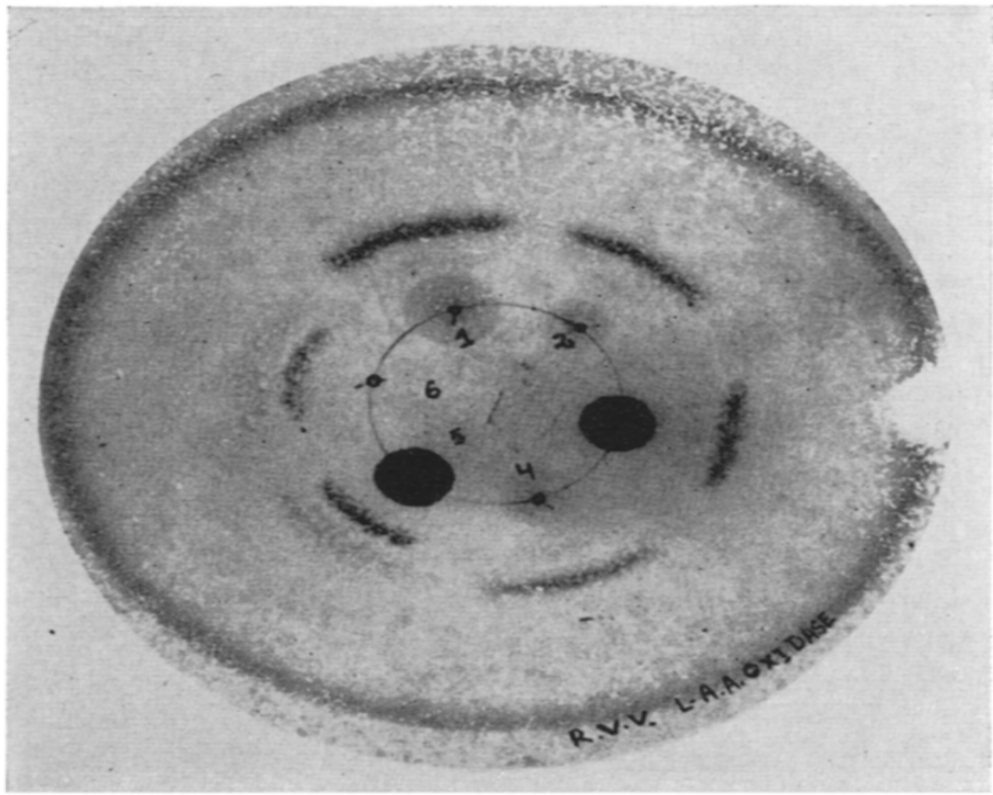

Fig. 1. The circular paper chromatogram showing the detection of L-amino acid oxidase in the venom of Russell's viper. Solvent = *n*-Butanol–acetic acid–water (4 : 1 : 5).

imidazole acetic acid and urocanic acid were used for spotting different volumes of those compounds on paper chromatograms. Circular paper chromatography was carried out according to the method of GIRI AND RAO[4].

Usually the reaction was carried out with 1 ml (2 mg/ml) L-histidine hydrochloride solution, 1.0 ml of 0.05 *M* Tris–HCl buffer pH 8.5 and 1.0 ml of 1 % solution of the venom under investigation. After incubation at 37° for 12–14 h, 10 μl aliquots of the reaction mixture were used for the chromatographic procedure. Two solvents were found to be useful for paper chromatography: solvent I = 77 % ethyl alcohol, solvent II = *n*-butyl alcohol–acetic acid–water, (4 : 1 : 5, v/v/v).

The spray reagent used was diazotized sulphanilic acid (Pauli's Reagent) prepared according to the method of MANN AND LEONE[5].

TABLE I

R_F VALUES OF L-HISTIDINE HYDROCHLORIDE, IMIDAZOLE ACETIC ACID AND UROCANIC ACID OBTAINED UNDER THE EXPERIMENTAL CONDITIONS DESCRIBED IN THE TEXT

	L-Histidine HCl	*Imidazole acetic acid*	*Urocanic acid*
Solvent I (77 % ethyl alcohol)	0.56	0.67	0.71
Solvent II (*n*-butyl alcohol–acetic acid–water) (4 : 1 : 5)	0.31	0.54	0.71

A typical chromatogram obtained is illustrated in Fig. 1.

The R_F values obtained for histidine, imidazole acetic acid and urocanic acid on a circular paper chromatogram using a 24 cm diameter disc of Whatman No. 1 filter paper are given in Table I.

With the solvents used, and especially with solvent II, imidazole acetic acid and urocanic acid can be distinctly separated from L-histidine hydrochloride. Thus even histidase which gives urocanic acid[6] with L-histidine as the substrate can be detected by this method in presence of the L-amino acid oxidase. As the D- and L-isomers of histidine have the same R_F values, even D-amino acid oxidase could be detected by the same procedure.

The colour of the bands for all three compounds is reddish brown on paper chromatograms. It is remarkably stable and the chromatograms can be preserved in the dark for months without any apparent fading of the bands. Of the several solvents tried to elute the coloured products from paper chromatograms, glass distilled water was found to be most satisfactory. 3 ml of glass distilled water could extract all the colour from the bands in 20–30 min. After elution of the colour from filter paper, the solution was yellowish red and could be measured colorimetrically.

Coloured complexes of all the three compounds had a maximum absorption at 360 mμ in the visible region of the spectrum as determined in a Unicam spectrophotometer Sp. 600. The original imidazole compounds had no absorption at this wave length. Hence the absorption of coloured products of both imidazole acetic acid and urocanic acid with Pauli's reagent can be measured and can be utilized for quantitative determination of L-amino acid oxidase as well as histidase. Imidazole acetic acid could be measured over a range of 5 to 40 μg and urocanic acid in the range of 5 to 60 μg under the conditions of the experiment described above.

As soon as the reaction mixture is applied to the paper chromatogram and dried, further enzymic reaction stops and hence the extent of oxidation of the substrate can be followed both qualitatively as well as quantitatively.

This method was routinely employed in our laboratory to detect and estimate L-amino acid oxidase in Indian snake venoms and also that in the white and yellow venoms of *Vipera ammodytes* after electrophoretic and chromatographic fractionation.

Another way of comparison of activities in venoms on a semiquantitative basis has been to compare the concentrations of venoms to produce a minimum visible reaction on paper chromatograms or the minimum concentrations of different venoms required to convert all the substrate into imidazole acetic acid.

DISCUSSION

As observed by paper chromatography, since L-amino acid oxidase converts all of L-histidine hydrochloride into imidazole acetic acid, the reaction can be used for the biological preparation of imidazole acetic acid following the procedure of MEISTER[7].

Histamine hydrochloride and L-histidine hydrochloride have the same R_F values in the *n*-butyl alcohol–acetic acid–water solvent. Hence if L-histidine decarboxylase is present in the reaction mixture, its presence will not interfere with L-amino acid oxidase activity determinations.

Though L-histidine is relatively less susceptible to the action of L-amino acid oxidase of snake venoms as compared to L-leucine[1], the method described in this

communication has the added advantage in that the product of reaction distinctly gets separated from the substrate on paper chromatograms, thus affording a quick identification of that enzyme qualitatively as compared to the method of BOMAN AND KALETTA[3], where the disappearance of the ninhydrin reacting spot is the criterion of the activity.

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SUMMARY

A paper chromatographic method is described for the detection and estimation of L-amino acid oxidase in snake venoms using L-histidine as the substrate. The product of reaction is separated paper chromatographically using 77% ethyl alcohol or *n*-butyl alcohol–acetic acid–water. The method can also be applied to the detection and determination of histidase and D-amino acid oxidase. Histidase and L-histidine decarboxylase do not interfere in the determination of L-amino acid oxidase.

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