薄層クロマトグラフィー・デンシトメトリーによるイソウロンおよびエナミノケトンの定量とHansenula saturnusによるイソウロンの分解過程

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Note

Determination of Isouron and Its Enaminoketone Metabolite by Thin Layer Chromatography-Densitometry in *Hansenula saturnus* Metabolism

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In our previous paper,[1,2] it was indicated that isouron was metabolized to 1-(1-amino-4,4-dimethyl-3-oxo-1-pentenyl)-3,3-dimethylurea (enaminoketone hereafter) through reductive cleavage of the isoxazole ring. The work was done by the soil perfusion technique. A strain of yeast responsible for this reaction was isolated and identified as *Hansenula saturnus*. Furthermore, the time-dependence of isouron degradation by cell suspension of the organism was studied qualitatively by thin layer chromatography. The quantitative results of these experiments were insufficient. Fluorometric,[3] colorimetric[4] and gas chromatographic[5] methods for the assay of isouron have been reported. These methods are accurate, but their time consumption is inconvenient if large numbers of samples have to be analyzed. In this study, a method of thin layer chromatography (TLC) and densitometry was applied to the direct determination of both isouron and its metabolite, enaminoketone, for the metabolism by microorganisms. The method was found to be simple and rapid. The degradation of isouron by *Hansenula saturnus* was analyzed by this method.

MATERIALS AND METHODS

1. *Chemicals*

Isouron [3-(5-*tert*-butyl-3-isoxazolyl)-1,1-dimethylurea] and 1-(1-amino-4,4-dimethyl-3-oxo-1-pentenyl)-3,3-dimethylurea (enaminoketone) were prepared as described in the previous paper.[2]

2. *Determination of Isouron and Enaminoketone by Thin Layer Chromatography and Densitometry*

Silica gel G plate with fluorescent indicator (Merck, Art. 5715) were used for the separation of isouron and enaminoketone. Ten μl of the sample was spotted on the plate, and the plate was developed upward with chloroform–acetone (30 : 5). Filter paper moistened with the same solvent was put on the inner walls of the developing cabinet. Development of the plate up to a height of 8 cm (corresponding to 13–15 min developing time) was sufficient for a reproducible separation of isouron and enaminoketone. The feasibility of the separation on the TLC plate is demonstrated by the profile shown in Fig. 1. The spot of enaminoketone on the TLC was visualized by placing it under a UV-lamp. The quantitative determination of these compounds was achieved by using A Shimadzu-UV-Visible Chromatogram Scanner Model CS-900. The absorbance at the spot on the plate was measured by linear scanning at the wave length of 240 nm with reflection-absorption photometry. The scanning rate was 20 mm per min and the dimension of the light beam was 1.0×10.0 mm.

3. *Isouron Degradation by Cell Suspension of Hansenula saturnus*

H. saturnus was grown in a modified

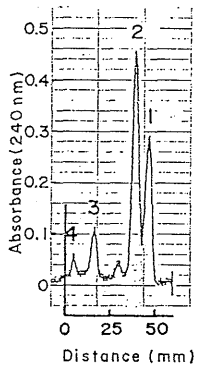

Fig. 1 TLC profile of isouron and enaminoketone.

1: Isouron, 2: Enaminoketone, 3: Metabolite B, 4: Metabolite C.
TLC conditions; Adsorbent: Silica gel G 60 (Merck, Art. 5715), Solvent: Chloroform–acetone (30 : 5), Developing time: 15 min, Sample volume: 10 μl (0.25–1.5 μg).
Densitometric conditions; Measuring mode: Reflection-absorption photometry, Wave length: 240 nm, Dimensions of light beam: 1.0×10.0 mm, Scanning rate: 20 mm/min.

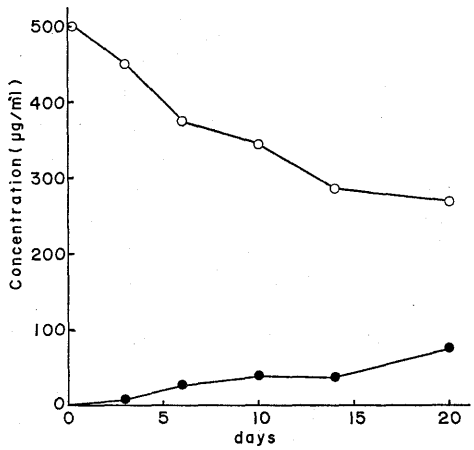

Fig. 2 Degradation of isouron by cell suspension of *Hansenula saturnus*.

○: isouron, ●: enaminoketone.
The cell suspension was incubated with 500 μg of isouron per ml at 28°C.

Sabourauds' agar medium (4.0% glucose, 1.0% polypeptone, 0.5% yeast extract and 1.5% agar) at 28°C for 3 days. The cells were collected and washed twice with 0.06 M phosphate buffer (pH 7.0). The washed cells were resuspended in the buffer to give 20 mg dry weight of cells per ml. Isouron was then added to the suspension, which was incubated at 28°C. One ml of the suspension was periodically collected for the determination of isouron and enaminoketone.

Table 1 *Rf* values of isouron and enaminoketone in various solvent systems.

Solvent	*Rf* values	
	Isouron	Enaminoketone
Ethyl ether	0.34	0.37
Ethyl ether–hexane (7 : 3)	0.20	0.24
Isopropyl alcohol–hexane (1 : 1)	0.64	0.70
Chloroform–acetone (1 : 1)	0.53	0.48
Chloroform–acetone (30 : 5)	0.54	0.47
Chloroform–methyl alcohol (9 : 1)	0.48	0.43
Chloroform	0.36	0.32

TLC conditions were the same as those shown in Fig. 1.

4. Extraction of Isouron and Enaminoketone

The cell suspension collected was centrifuged at 1500 *g* for 15 min. The supernatant was separated, saturated with NaCl, and one ml of ethyl acetate–benzene (3 : 1) was added to it. The entire sample was stirred for a few minutes by a mixer and centrifuged again at 1500 *g* for 3 min. The organic solvent phase was collected. This extraction procedure was repeated twice. The total extracts were combined and concentrated to dryness by rotary evaporator under reduced pressure. The residue was dissolved in one ml of the solvent mixture mentioned above and ten μl of the solution was subjected to TLC using a microsyringe.

RESULTS AND DISCUSSION

Two solvents and six solvent mixtures were evaluated for their capability of separating isouron and enaminoketone on TLC plates (Table 1). The solvent mixture of chloroform–acetone (30 : 5) was found to be best suited for this purpose and was chosen for further studies. As indicated in Fig. 1, the spot of

enaminoketone was located below that of isouron, and could be recognized with UV-lamp. The absorption maximum of isouron was at 239 nm, whereas those of enaminoketone were at 240 nm and 305 nm. The absorbance of both compounds on the plate could be measured simultaneously by scanning from the front edge to the starting point at a wave length of 240 nm. The calibration curves of isouron and enaminoketone were linear in the range of 0.25–1.5 μg/spot according to the regression equations $y=0.176x+0.03$ for isouron and $y=0.176x+0.02$ for enaminoketone. The detection limits of these compounds were 0.05 μg. The correlation ratio determined by the least-squares method was more than 99% for both curves. The coefficients of variation for the determination of isouron and enaminoketone amounted to the averaged values of 2.5% and 3.3% ($n=5$), respectively. By repeated determinations of sample solutions at a constant concentration (100 μg/ml) by this method, the overall recoveries of isouron and enaminoketone were 92.3±3.43% and 91.8±4.06% ($n=10$), respectively. The result indicated that the assay outlined above can be used as a simple and rapid method to analyze large numbers of samples.

The degradation of isouron to enaminoketone by a cell suspension of *H. saturnus* was studied quantitatively by this assay. The result is shown in Fig. 2. Isouron was metabolized gradually and 40% of the initial concentration disappeared after 20 days of incubation. Enaminoketone was formed as the main metabolite in the cell suspension while isouron was degraded, and the concentration of the metabolite was increased to 75 μg/ml. The results indicated that the time-dependence of isouron degradation by *H. saturnus* could be analyzed quantitatively with the assay by thin layer chromatography and densitometry, and the organism has the capability of degrading isouron to enaminoketone by reductive cleavage of the isoxazole ring.

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要　　約

薄層クロマトグラフィー・デンシトメトリーによるイソウロンおよびエナミノケトンの定量と *Hansenula saturnus* によるイソウロンの分解過程

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薄層クロマトグラフィー(TLC)とリニアスキャンニング方式の TLC スキャナーを用いるデンシトメトリーによって除草剤イソウロンとその代謝物エナミノケトンの分離と定量を行なった．吸着剤としてシリカゲルを用い，クロロホルム-アセトン (30:5) の混合溶媒で展開すると両化合物は良好に分離した．これを TLC スキャナーを用いて波長 240 nm における吸光度で測定すると両化合物の量と吸光度の間には 0.25～1.5 μg/spot の範囲で直線関係が成立した．この定量法を用いて *Hansenula saturnus* によるイソウロンの分解を調べた．イソウロンは徐々に分解し 20日目には初濃度の40%が消失した．それに対応してエナミノケトンが生成し培地中に蓄積した．