PRO EXPERIMENTIS

A convenient thin layer chromatographic method for the detection of cyclochlorotine and simatoxin, toxins from *Penicillium islandicum* Sopp.[1]

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Summary. A rapid, convenient TLC method is described for detection of cyclochlorotine, a carcinogen produced by the commonly found storage mold *Penicillium islandicum* Sopp. This method has also led to the isolation of a new toxic metabolite, simatoxin.

The commonly found storage mold *Penicillium islandicum* Sopp has been identified as the mold responsible for causing the so-called 'yellow rice toxicosis'[2-4]. Isolates of this fungus have been obtained from a wide variety of foodstuffs, including rice[2,4], peanuts[5] and soybeans[6]. A large number of toxic and non-toxic metabolites have been isolated from this mold. Potent hepatotoxic activity has been observed for the cyclic polypeptides cyclochlorotine[7,8] and islanditoxin[9,10], both isolated in 1955.

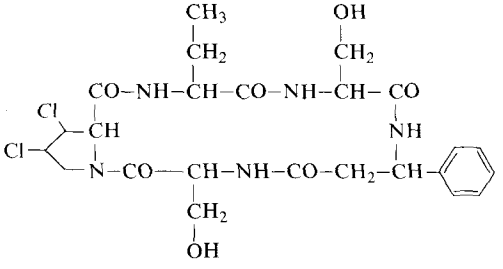
Physicochemical properties of the 2 peptides are almost identical. However, no direct comparison between the 2 toxins has yet been made. In the past, research on these water-soluble, labile polypeptides has been seriously hampered due to their low yield and lack of suitable detection techniques. Cyclochlorotine has been isolated from the culture filtrate of *P. islandicum* in a yield of 0.4–5 mg/l of the filtrate[2,11]. Long term feeding studies with mice have shown that this toxin (LD_{50} 0.475 mg/kg, in mice, s.c. administration) induces liver carcinoma causing violent symptoms such as necrosis and vacuolation of liver cells[12,13].

A previous method for the determination of cyclochlorotine involved a combination of adsorption of the crude peptide on charcoal, gel filtration, ammonolysis and ultraviolet photometry[14]. We report here a convenient detection method for the toxin, based upon the color reaction of the peptide with chlorine-o-tolidine reagent[15,16].

Material and methods. Penicillium islandicum strain WF-18-12 (obtained through the courtesy of Professor Y. Ueno, Science University of Tokyo, Tokyo) was used for the production of the toxin. Liquid fermentations[11,17] using modified Wickerham medium as well as solid state fermentations using various grains were carried out[17]. In grain fermentations, glutinous rice, brown rice, long grain rice, white wheat, red wheat and rye were used as substrates. In a typical experiment, red wheat (300 g) was placed into a Fernbach flask (2.8 l, wide neck) and moistened with water (150 ml). After autoclaving, the substrate was inoculated with 1 moldy grain from a rice culture of *P. islandicum* and incubated on a rotary shaker for 14 days at 30 °C. The contents of the flask were extracted with methylene chloride. After separation of the solvent from the residual grain, the latter was extracted with water, the aqueous solution was treated with charcoal (Darco S-51, obtained from ICI America), filtered and the charcoal extracted with acetone-water (1:1). After filtration, the charcoal was treated with n-butanol and n-butanol solution extracted with 1 M phosphate buffer (pH 7.0). The n-butanol solution, upon concentration provided the crude cyclochlorotine. It was chromatographed on a Sephadex LH 20 column, using water-methanol (1:1) as the solvent. Fractions (5 ml) were collected and examined by TLC using precoated plates (5×20 cm silica gel F-254 plates, thickness 0.25 mm, EM Reagents) and n-butanol:acetic acid:water (4:1:2) as the solvent system. They were dried and exposed to chlorine gas for 5 min inside a glass chamber. Excess chlorine was allowed to evaporate and the plate was sprayed with o-tolidine reagent (160 mg o-tolidine, 30 ml acetic acid, 470 ml water and 1 g potassium iodide)[15,16]. A bright yellow spot (Rf 0.7) was observed for fractions containing cyclochlorotine. The identification of the peptide as cyclochlorotine was confirmed by high resolution mass spectroscopic studies[18]. A 2nd yellow spot (Rf 0.4) indicated the presence of the new toxin, simatoxin.

Results and discussion. Cyclochlorotine, a colorless solid (m.p. 254–5 °C) is produced by *P. islandicum* Sopp in a low yield. The peptide is very labile and contains a rather unusual cis-dichloroproline unit in its structure. On treatment with mild alkali, it undergoes facile dehydrochlorination and the resulting pyrrole has a strong absorption at 268 nm (ε 18,000). This property has been used by Ueno and coworkers[14] in estimating the purity of cyclochlorotine. However this method is time-consuming and non-specific. The major significance of the present method is that it provides a rapid technique for the detection of the cyclochlorotine. The detection limit of purified toxin is approximately 1 ppm.

This thin layer procedure has also led to the detection of a 2nd spot at Rf 0.4 which was attributed to a hitherto unknown toxic metabolite, which we call simatoxin[17]



Cyclochlorotine

(LD_{50} < 10 mg/kg, in new born rats, i.p.). Structural elucidation of this compound is in progress.

Our studies have shown that a variety of common foodgrains including glutinous rice, brown rice, red wheat, white wheat, white corn, soybean flakes and rye support the production of cyclochlorotine by *P. islandicum* under laboratory conditions[17]. However, there has been no evidence so far about the actual occurrence of this toxin in foodstuffs. Application of our detection method should prove useful in determining whether cyclochlorotine is actually produced during the storage period of these common foodgrains and constitutes a potential human health hazard.

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Préparation des sous-unités de la dopamine β hydroxylase humaine et des anticorps anti-sous-unités
Preparation of human dopamine β hydroxylase subunits and antibodies against these subunits

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Summary. The present study deals with the dissociation of human dopamine β hydroxylase (DBH) in subunits with a mol. wt of 79,500, and the preparation of specific antibodies in rabbits. No cross-reactivity was observed between human DBH and antibodies against human DBH subunits.

La dopamine β hydroxylase (EC 1.14.17.1) est liberée pendant la stimulation du système nerveux sympathique, simultanément avec l'adrenaline et la noradrenaline[3-5] et la mesure du taux sanguin de cette enzyme est un bon index du fonctionnement de ce système nerveux[6]. La détermination de l'activité enzymatique plasmatique peut être une aide dans le diagnostic et le pronostic de certaines maladies, en particulier dans les tumeurs de la crête neurale où on a observé des variations importantes de cette activité[7,8].
Wallace[9] rapporte que la dopamine β hydroxylase est une glycoprotéine tétramérique. Des méthodes de dissociation de la DBH bovine et la structure des sous-unités ont été décrites[10,11]; les auteurs ont noté que les anticorps contre la DBH native ne précipitent pas les sous-unités.
Nous avons pensé que, dans certaines conditions pathologiques, des sous-unités de l'enzyme peuvent être libérées dans le sang. Les sous-unités n'ayant pas d'activité enzymatique, il convenait de mettre au point une méthode de dosage radioimmunochimique, nécessitant l'utilisation d'anticorps spécifiques. Nous décrivons ici la méthode de dissociation de la dopamine β hydroxylase d'origine humaine et l'induction d'anticorps spécifiques chez le lapin.
Matériels et méthodes. L'absence de réaction croisée entre la dopamine β hydroxylase d'origine humaine et la DBH bovine nous obligeant à utiliser des anticorps homologues, nous avons préparé l'enzyme à partir d'un pheochromocytome humain, selon le méthode décrite par Aunis[12]. L'enzyme est dissociée en 4 sous-unités identiques par traitement de la protéine native par la dithiothreitol (50 mM) et chauffage à 60 °C pendant 30 min.
La pureté de la protéine native est vérifiée par électrophorèse sur gel de polyacrylamide selon la méthode de Davis[13]. Les sous-unités sont soumises à une électrophorèse sur gel selon Weber-Osborn[14], en même temps que plusieurs protéines marqueurs de poids moléculaires connus: inhibiteur de trypsine, la serum albumine bovine et la RNA polymérase d'*E. coli* (Combithek Boehringer).
Pour les immunisations initiales, les lapins reçoivent à 15 jours d'intervalle 3 injections de 1 mg de DBH humaine en émulsion dans 1 ml d'adjuvant complet de Freund. Les injections sont pratiquées dans les coussinets des pattes de l'animal. Les injections ultérieures contenant 0,3 mg de protéines sont faites par voie s.c. multiple, 1 fois par mois.
Les injections de la DBH dissociée (0,3 mg) sont effectuées selon le même protocole. Le sang des lapins est prélevé 1 semaine après la 3ème injection ou 1 injection de rappel, par ponction cardiaque. Le sérum est recueilli et conservé à −20 °C.
Des dilutions d'antisérum sont analysées en présence des antigènes, par immunodiffusion sur gel d'agar à 1,5% (Outcherlony) pendant 3 jours à +4 °C.

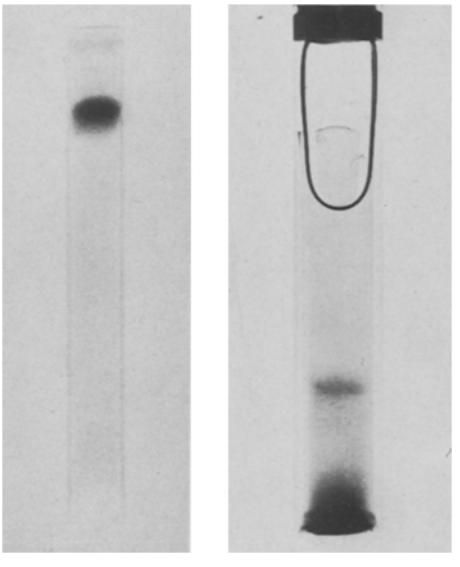

Fig. 1. Electrophorèse sur gel de polyacrylamide de la dopamine β hydroxylase humaine purifiée à gauche et de la dopamine β hydroxylase dissociée (à droite). Environ 40 µg de protéines dans un volume de 100 µl sont soumis à l'électrophorèse.