Quantification of the Particle Method for Chemotactic Bioassay Using Peronosporomycete Zoospores

Tomohiko Takayama, Yukiharu Fukushi, Md. Tofazzal Islam, and Satoshi Tahara*

Laboratory of Ecological Chemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan. Fax: +81-11-706-4182. E-mail: tahara@abs.agr.hokudai.ac.jp

* Author for correspondence and reprint requests

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We estimated the amount of test solution absorbed by each Chromosorb W AW particle (60–80 mesh) using an isotopic technique to quantitate the particle method. ^{14}C-Labeled standard compounds like carbendazim (MBC), 5-*O*-methylcochliophilin A, sucrose and proline were dissolved in several solvents, and Chromosorb carrier particles were treated with the solution to coat the particle with these test compounds. The ratios of the radioactivity of 5 μl of the test solution to that of 2 mg of carrier particles treated with the solution at some different concentrations were measured. It was found that each carrier particle holds approx. 3.8 nl of the test solution within a range of 2×10^{-3} to 1×10^{-7} M concentrations. Accordingly, it is now possible to widely use the particle method as a quantitative procedure to assay chemotaxis of Peronosporomycete zoospores.

Key words: Peronosporomycete Zoospore, Chemotaxis, Particle Method

Introduction

Aphanomyces cochlioides Drechsler, a soilborne phytopathogen belonging to Leptolegniaceae (Peronosporomycete: Saprolegniales), is responsible for a root rot disease of spinach (*Spinacia oleracea* L.) and a damping-off disease of sugar beet (*Beta vulgaris* var. *rapa* Dum.) (Ui and Nakamura, 1963; Scott, 1961). The bi-flagellated zoospores of *Aphanomyces* spp. can swim through soil water to find roots of host plants, which have been believed to be mediated by host-specific chemical signals (Rai and Strobel, 1966; Yokosawa *et al.*, 1974). Compounds such as indole-3-carbaldehyde from cabbage seedlings (Yokosawa and Kuninaga, 1979), prunetin from pea seedlings (Yokosawa *et al.*, 1986), and cochliophilin A (**1**) from the roots of spinach (Horio *et al.*, 1992) have been identified as the host specific zoospore attractants for zoospores of *A. raphani*, *A. euteiches*, and *A. cochlioides,* respectively. In contrast to susceptible (host) plants, nonhost plants may exude chemical signals which in some way contribute to resistance (Mizutani *et al.*, 1998; Tahara *et al.*, 1999). Therefore, it is mandatory to establish a convenient bioassay system to detect chemotactic substances regulating the behavior of zoospores quantitatively as well as qualitatively.

Three methods have been reported so far for chemotactic assay of zoospores: (A) the capillary method (Khew and Zentmyer, 1973); (B) the particle method (Horio *et al.*, 1992); and (C) the drop method (Müller, 1976; Takayama *et al.*, 1998), with some variations in each method. The capillary method was applied to isolate indole-3-carbaldehyde and prunetin (Yokosawa and Kuninaga, 1979; Yokosawa *et al.*, 1986). The drop method has first been developed by Müller (1976) for evaluation of sexual chemotaxis in marine brown algae. Takayama *et al.* (1998) have applied this method to quantify the potency of cochliophilin A (**1**) dissolved in a perfluorocarbon droplet.

The particle method has been devised by Horio *et al.* (1992) as a convenient bioassay method to observe the behavior and/or the morphological change of *A. cochlioides* zoospores around particles of Chromosorb W AW (for Gas-Liquid Chromatography) coated with a crude plant extract or a test compound and dropped in the zoospore suspension under a light microscope. This method is very simple and rapid to get the results and needs only micromolar levels of test compounds/extracts for the bioassay. It was successfully applied to bioassay-guided isolation of a highly potent host-specific attractant cochliophilin A (**1**) in the roots of spinach (Horio *et al.*, 1992). The response of zoospores toward cochliophilin A coated particles was identical to their response toward host roots (Islam *et al.*, 2003), which offers

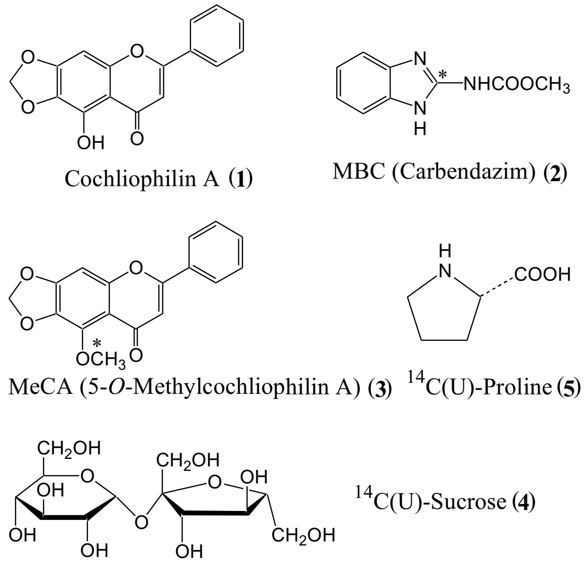

Fig. 1. Structures of chemical compounds used for quantification of the 'particle method' using an isotopic technique.

an opportunity to use Chromosorb particles as the dummy of roots to evaluate the activity of plant secondary metabolites toward pathogenic zoospores. However, the method is qualitative in nature and the investigator will not know the absolute amount of each test material held on the porous particle. Furthermore, it is not clear whether the amount of each test compound held on the particle is proportional to the concentration of the test solution or not, and whether the effects of solvent polarity on the amounts held on the particle are negligible or not.

In the present study, we conducted an investigation for quantification of the particle method by using some ^{14}C-labeled standard compounds with a wide range of polarity, like carbendazim (MBC, **2**), 5-methoxy-6,7-methylenedioxyflavone [5-*O*-methylcochliophilin A (MeCA), **3**], sucrose (**4**) and proline (**5**) (Fig. 1) in different solvents and estimated the amount of the test solution absorbed by each celite particle when coated with chemical compounds for the bioassay.

Materials and Methods

Chemicals

[2-^{14}C]-Carbendazim (**2**), 98.1 Mbq/mmol, and [5-O^{14}CH$_3$]-5-methoxy-6,7-methylenedioxyflavone (5-*O*-methylcochliophilin A, **3**), 95.9 Mbq/mmol, were prepared in our laboratory (unpublished). [^{14}C(U)]-Sucrose (**4**), 22.2 Gbq/mmol, in EtOH/H_2O (2:98) and [^{14}C(U)]-proline (**5**), 10.3 Gbq/mmol, in EtOH/H_2O (2:98, v/v) were purchased from Moravek Biochemicals Inc., CA, USA and New England Nuclear Research Products, MA, USA, respectively. Compounds **2**, **3**, **4** and **5** (Fig. 1), respectively, were dissolved at 2.0×10^{-3} M, 1.0×10^{-3} M, 1.6×10^{-6} M, and 7.2×10^{-5} M in EtOAc, acetone, MeOH and MeOH, and each solution was diluted to give a series of ⅓ and ⅒ dilution in each solvent, namely, compound **2** in EtOAc, acetone and MeOH; compound **3** in EtOAc; sucrose (**4**) and proline (**5**) in MeOH.

Carrier particles

Commercially available Chromosorb W AW (60–80 mesh) (Fig. 2) (Advanced Mineral Co., CA, USA) was successively washed with EtOAc, acetone, MeOH and water, and dried in a desiccator followed by sieving to remove smaller particles.

Coating carrier particles with the standard solution

As a standard method, the carrier particles (2 mg) were put on a watch glass and 20 μl of a standard solution was dropped onto the particles. Excess solution on the watch glass was immediately absorbed with a piece of filter paper, and

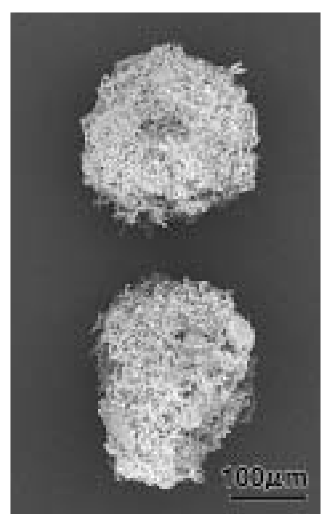

Fig. 2. Scanning electron micrograph of two Chromosorb W AW particles.

the particles were allowed to dry in air at room temperature for a few minutes.

Determination of radioactivity

To 5 ml of scintillator Aquasol-2 (New England Nuclear Research Products, NEF-952) in a Wheaton counting vial no. 986542 was added 5 μl of the standard solution containing a ^{14}C-labeled standard compound at a set concentration in the solvent mentioned or 2 mg of carrier particles treated with the standard solution (10 μl/mg), and the disintegration per min (DPM) was measured by a scintillation counter (ALOKA LSC-5100) in the usual way.

Results and Discussion

^{14}C-MBC (**2**) was dissolved in EtOAc, acetone and MeOH, respectively, using 2×10^{-3}, 1×10^{-3}, 6.67×10^{-4}, 1×10^{-4}, 6.67×10^{-5}, 1×10^{-5}, 6.67×10^{-6} and 1×10^{-6} M, and then the radioactivity (disintegration per min) of 5 μl per sample was measured in Aquasol-2 by a scintillation counter. The resulting DPMs plotted in Fig. 3 (solid lines) show that DPMs in each solution for a set concentration are nearly the same even in a different solvent and are proportional to the concentration of the standard solutions. The DPMs of the dotted lines in Fig. 3 show the DPMs of 2 mg of carrier particles treated with each standard solution. These plots revealed to be located on a band with a narrow width and parallel to the solid lines in a range of 1×10^{-6} to 2×10^{-3} M of MBC. These results suggest that the DPMs due to 2 mg of carrier particles treated with standard solutions (10 μl/mg carrier) are barely affected by the solvent species, but depend on the concentration of the standard solution used to coat the carrier particles. Thus we could calculate a volume of the test solution held on carrier particles (μl/mg), according to the following equation: Holding volume of test solution of the carrier (μl/mg) = (DPMs due to 5 μl of the standard solution at a given concentration)/[DPMs on 2 mg of carrier particles treated with the same standard solution (10 μl/mg)].

The holding volumes at a given concentration for MBC (**2**) in EtOAc, acetone and MeOH were calculated and averaged (Table I). These tests were applied to MeCA (**3**) in EtOAc, sucrose (**4**) and proline (**5**) in MeOH. It was found that in principle the results were the same as those for MBC in Fig. 3. Holding volumes of carrier particles (μl/mg) for four standard compounds in three solvents are summarized in Table I. There was no significant difference among holding volumes of carrier particles treated with MBC dis-

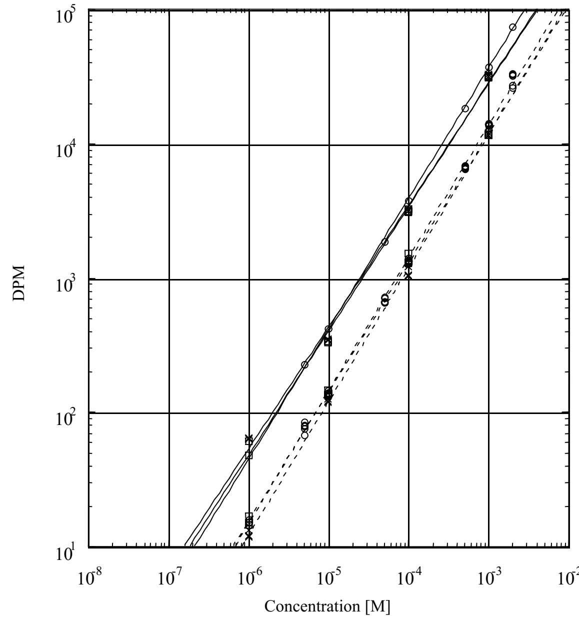

Fig. 3. Radioactivities (disintegration per min = DPM) of ^{14}C-MBC standard solution (DPM/5 μl) in EtOAc, acetone or MeOH and those held in 1 mg of Chromosorb W AW (carrier) particles. —○— : DPM of EtOAc solutions (5 μl each); —□— : DPM of carrier particles (1 mg each) treated with EtOAc solutions; —×— : MeOH solutions; - - ○ - - DPM of carrier particles (1 mg each) treated with EtOAc solutions; - - □ - - DPM of carrier particles treated with acetone solutions; - - × - - DPM carrier particles treated with MeOH solutions. Each experiment was repeated at least 6 times and results were averaged (see also Table I).

Table I. Calculated volumes of the test solutions held on the carrier particles (Chromosorb W AW, 60–80 mesh).

^{14}C-labeled compound/solvent	Solution held on the carrier [μl/mg]* EtOAc	Acetone	MeOH
MBC	1.86 ± 0.139	2.00 ± 0.198	1.82 ± 0.161
MeCA	1.90 ± 0.220	NT	NT
Sucrose	NT	NT	1.67 ± 0.124
Proline	NT	NT	2.46 ± 0.224

NT: not tested.
* Results are based on at least six repetitions using more than six solutions differing in the concentration (MBC) in three solvents, respectively. Others have been dissolved in one solvent, MeCA in EtOAc, and sucrose and proline in MeOH.

solved in EtOAc, acetone and MeOH, or measuring MeCA solutions with EtOAc. However, results of sucrose and proline solutions in MeOH were a little different from the former values, even though the deviation was less than 30%. In our bioassay the sample solution was usually diluted stepwise by ⅓ or ⅒, therefore deviation around 30% is not so serious.

Thus we conclude about our particle method that the amount of the compounds with a wide range of polarity in solvents held on the carrier particles was equivalent to *ca.* 1.90 μl of the test solution per milligram of carrier in a wide range of concentration (1×10^{-8} to 1×10^{-3} M).

To know the number of carrier particles in a given sample, approx. 1 mg of Chromosorb W AW (60–80 mesh) (Fig. 2) was correctly weighed and the particle numbers were counted ten times. The counting revealed that 1 mg of the used Chromosorb W AW consisted of 498 ± 23 particles. Together with the holding volume of the standard solution and average numbers of carrier particles in 1 mg we are able to estimate the holding volume of the standard solution per one particle as *ca.* 3.8 nl. Thus we could quantify the particle method, namely, roughly 4 nl of a test solution is present in each carrier particle.

The pathogenic zoospores of *Aphanomyces cochlioides*, a cause of root rot of *Spinacia oleracea* and some other Chenopodiaceae and Amaranthaceae are guided to host plants by the host-specific attractant released from the roots and establish the infection. In order to detect the zoospore responses to chemotactic agents in the zoospore suspension, it is essential to generate a concentration gradient of the relevant agents (Islam *et al.*, 2003). The particle method is very simple and performed in a very small scale, whereas the method has been qualitative in principle. However, the present study gives us a quantitative aspect of this method, since now it is possible to calculate the amount of the sample on each particle, that is, *ca.* 3.8 nl equivalent of the test solution. Cochliophilin A (**1**) is a very potent attractant towards the zoospores which are attracted to a carrier particle coated with a 1×10^{-9} M solution. In this case, the amount of the attractant held on the particle is estimated to be approx. 1×10^{-15} g.

As reported in our previous papers, the particle method enables us to isolate not only attractants, like cochliophilin A (**1**) in spinach roots (Horio *et al.*, 1992), *N-trans*-feruloyl-4-*O*-methyl-dopamine (Fig. 4) in *Chenopodiun album* roots (Horio *et al.*, 1993), and 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone in spinach leaves (Tahara *et al.*, 2001), but also zoospore motility inhibitors, like a mixture of 1-linoleoyl-2-lysophosphatidic acid monomethyl ester and *N-trans*-feruloyl-tyramine in *Portulaca oleracea* (Mizutani *et al.*, 1998) and nicotinamide in *Amaranthus gangetica* (Shimai *et al.*, 2002), and repellants, like medicarpin

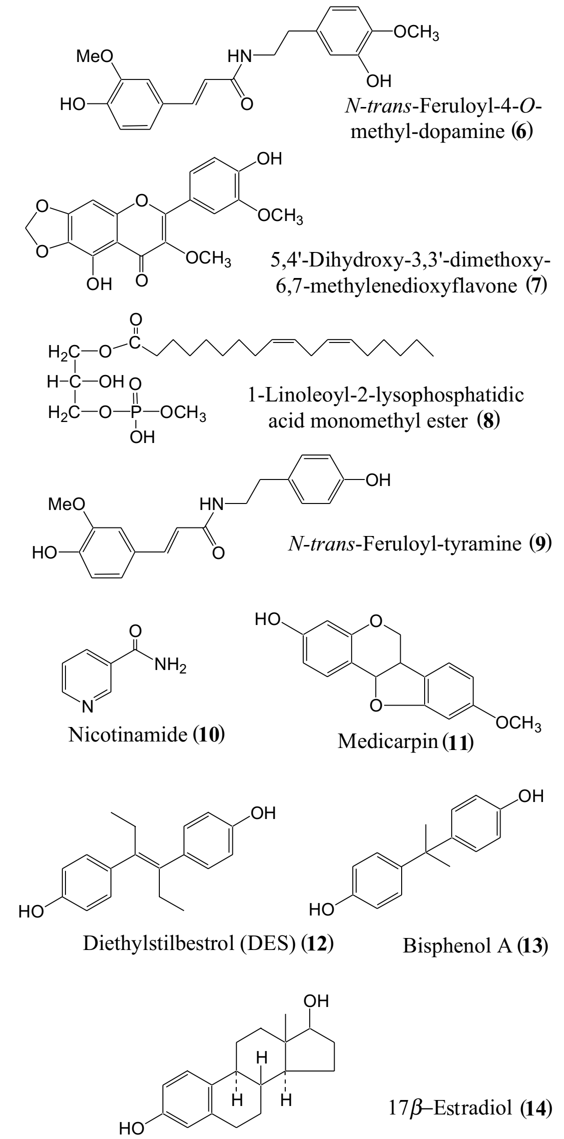

Fig. 4. Structures of some physiologically active compounds toward Peronosporomycete zoospores detected and evaluated by particle method.

in *Dalbergia odorifera* (Islam and Tahara, 2001b). Interestingly, the particle bioassay using the synthetic estrogen DES or the environmental pollutant bisphenol A, in addition to the natural estrogen 17β-estradiol showed a strong repellent activity against the zoospores (Islam and Tahara, 2001a). The structure-activity relationship between cochliophilin A-related flavones was also studied by the aid of the particle method (Kikuchi *et al.*, 1995). Furthermore, some natural products showed zoospore cell lytic activity around the carrier particles coated with them, for example, condensed tannins from *Lannea coromandelica* (Islam *et al.*, 2002) and anacardic acids from *Ginkgo biloba* (Begum *et al.*, 2002). Further information about this method is also found in our reviews (Tahara and Ingham, 2000; Islam and Tahara, 2001b).

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