Journal of Chromatography, 628 (1993) 122–126
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 695

Short Communication

Determination of kinetin in callus of *Panax ginseng* by liquid chromatography

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(First received July 2nd, 1992; revised manuscript received October 19th, 1992)

ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed for the determination of kinetin levels in *Panax ginseng* dried callus, fresh callus and culture media. Ground dried callus was suspended in borate buffer and extracted with ethyl acetate. The extract was eluted through a cation-exchange column (Amberlite CG-50), then re-extracted with ethyl acetate. This extract was subjected to HPLC. Kinetin levels were determined by gradient elution on an Inertsil ODS-2 column and UV detection at 280 nm. The ion-exchange column chromatographic purification step could be eliminated with kinetin extracts from fresh callus and culture media. The recovery of kinetin from dried callus spiked at 5 $\mu g\ g^{-1}$ was 72.0% and those from fresh callus and media spiked at 1.0 and 0.5 $\mu g\ g^{-1}$ were 72.8 and 84.2%, respectively. Kinetin was not detected in dried callus of *P. ginseng*.

INTRODUCTION

Large numbers of useful substances such as food additives and medicinal drugs can be produced by plant tissue culture. It has been reported that *Panax ginseng* callus, coded Pg-1, produces almost the same pharmacologically active saponins and ginsenosides as cultivated ginseng root [1–4].

Recently interest has been increasing in the concentrations of residual plant growth regulators from callus, including that in growth medium. Analyses of plant hormones from cultures have generally been carried out by gas chromatography (GC) [5–7], GC–mass spectrometry (MS) [8,9] and high-performance liquid chromatography (HPLC) [10,11].

Kinetin has been determined after permethylation, using GC with nitrogen–phosphorus [6] or MS detection (single-ion monitoring) [9], or after trifluoroacetylation [7] or trimethylsilylation [5] using GC with electron-capture detection. These methods

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0021-9673/93/$06.00

suffer several problems related to clean baseline separation of chromatograms, conciseness and reproducibility, owing to the similarity of the structures of kinetin and of nucleic acids orginally contained in the callus, and the low kinetin concentration. Various HPLC procedures have been applied to the determination of kinetin in recent years [10,11], but only model systems and not real samples were assayed.

The purpose of this investigation was to develop a sensitive method for the determination of kinetin in *Panax ginseng* callus to establish whether kinetin is taken up by fresh and dried callus from the medium.

EXPERIMENTAL

Materials

Panax ginseng C. A. Mayer callus was cultivated by Furuya *et al.*'s method [12]. Selected Pg-1 callus was transplanted on to modified Murashige and Skoog's medium containing 2 $\mu g\ g^{-1}$ of indole-3-butyric acid and 0.1 $\mu g\ g^{-1}$ of kinetin. The callus was maintained at 25°C in the dark, then subcultured and harvested at 4-week intervals. The harvested callus was dried under a stream of warm air at 40–50°C until the water content was reduced to 3%.

Reagents

Kinetin of biochemical grade was obtained from Wako (Osaka, Japan) and Amberlite CG-50 Type I from Orugano (Tokyo, Japan). Other reagents were of special grade, except acetonitrile, which was of analytical-reagent grade, from Wako.

Apparatus

A Model 880-PU pump, Model 7125 injector, Model 870 UV detector (Japan Spectroscopic, Tokyo, Japan) and Chromatopac C-R5A integrator (Shimadzu, Kyoto, Japan) were used, and identifications were made using HP1090A HPLC systems with UV photodiode-array detection (DAD) (Yokogawa Electric, Tokyo, Japan).

The analytical HPLC gradient flowed at 1.0 ml min^{-1} on an Inertsil ODS-2 HPLC column (250 × 4.6 mm I.D.; 5-μm diameter particles) (GL Science, Tokyo, Japan) at 30°C. A mixture of water and 95% acetonitrile containing 0.1% of trifluoroacetic acid was programmed from 97:3 to 65:35 (v/v) over 20 min, monitoring the wavelength at 280 nm. The injection volume was 20 μl. DAD was used for kinetin identification. The wavelength ranged from 210 to 350 nm.

Extraction and purification of kinetin from P. ginseng dried callus

A 1-g amount of dried callus was homogenized in 20 ml of 1 *M* borate buffer (pH 8.5), to which 5 g of sodium chloride were added. The homogenate was mixed well, then extracted with 50 ml of ethyl acetate three times.

The extracts were combined and concentrated to dryness, then the residue was dissolved in 20 ml of 0.01 *M* hydrochloric acid (sonicated for 15 min). A 20-ml volume of acetate buffer (pH 5.6) was added and the solution was purified on an ion-exchange column. The Amberlite CG-50 cation-exchange resin was washed with five column volumes of 1 *M* ammonia solution until the water layer became clear, then with water until the resin became neutral. The column was washed with five volumes of 1 *M* hydrochloric acid. Finally, it was washed with water until the pH stabilized between 5 and 6. The treated resin was suspended in 0.1 *M* acetate buffer (pH 5.6), then packed into a glass column of 1.6 cm I.D. to a height of 4 cm. The packed column was prewashed with 20 ml of 0.1 *M* acetate buffer (pH 5.6). The sample was applied to the column and drained at a flow-rate of about 2–3 ml min^{-1} until the surface of the resin was just covered, then the column was washed with 25 ml of 0.025 *M* acetate buffer (pH 5.6) followed by 1 *M* ammonia solution. The first 20 ml of ammonia eluate was drained and kinetin was eluted in the second 20 ml. This eluate was neutralized with 6 *M* hydrochloric acid, mixed with 1 *M* borate buffer (pH 8.5), then kinetin was re-extracted with 50 ml of ethyl acetate three times. The extract was concentrated to dryness and the residue was dissolved in 2 ml of 0.1% trifluoroacetic acid and analysed by HPLC.

Extraction of kinetin from P. ginseng fresh callus

Fresh callus (2.5 g) was chopped into small pieces and added to 5 ml of 0.1 *M* hydrochloric acid and homogenized. The homogenized sample was clarified by vacuum filtration and the residue was re-extracted as above. The filtrates were pooled and 5 ml of 1 *M* borate buffer (pH 8.5) and 5 g of NaCl

were added. Kinetin was extracted with 50 ml of ethyl acetate three times, the extracts were pooled and evaporated to dryness, then the residue was dissolved in 2 ml of 0.1% trifluoroacetic acid.

The ion-exchange column chromatographic purification step could be eliminated when analysing fresh callus.

Extraction of kinetin from the culture medium

A 5-ml volume of 1 *M* borate buffer (pH 8.5) and 5 g of sodium chloride were added to 10 g of medium and kinetin was extracted with ethyl accetate using the same method as described for fresh callus.

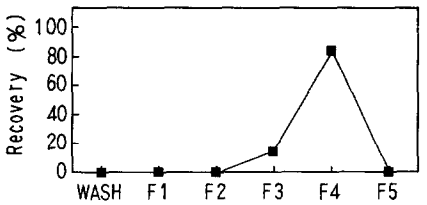

Fig. 1. Stepwise elution of kinetin from ion-exchange column. Resin treated with 1 *M* ammonia solution and 1 *M* hydrochloric acid was packed into a glass column of 1.6 cm I.D. to a height of 4 cm. The packed column was prewashed with 15 ml of buffer, then kinetin (20 μg) was applied. The column was washed with 25 ml of buffer and kinetin was eluted with 1 *M* ammonia solution (■). Each fraction (F1–F5) was 10 ml.

RESULTS AND DISCUSSION

Extraction of kinetin from aqueous solution

When 2 g of standard kinetin were added to 20 ml of 1 *M* borate buffer with 5 g of sodium chloride, then extracted with 50 ml of ethyl acetate three times, the recovery was 98%.

Purification by cation-exchange chromatography

The ethyl acetate extract of *Panax ginseng* callus contained numerous interfering compounds when determined by HPLC, and purification was necessary. A C_{18} cartridge and liquid–liquid extraction columns (Extrelut) were unsuitable for this process (data not shown). This was ascribed to the high concentration of pyrimidines and purines, which have similar structure and polarity to the kinetin in *Panax ginseng* cells.

Kinetin was isolated from autoclaved DNA by cation-exchange chromatography according to the method of Miller *et al.* [13]. First, we investigated the elution profile of kinetin from the Amberlite CG-50 column (2 × 1 cm I.D.) with hydrochloric acid. The column was prewashed with 1 *M* hydrochloric acid and water, then the applied kinetin was washed with 5 ml of 0.025 *M* acetate buffer (pH 5.6) and eluted with 25 ml of 1 and 2 *M* hydrochloric acid. However, when the resin was prewashed with 1 *M* ammonia solution, 1 *M* hydrochloric acid and water, in that order, the elution volume decreased. The capacity of the resin also decreased on pretreatment with ammonia solution. Therefore, the treated resin was packed into a column of 1.6 cm I.D. to a height of 4 cm to avoid a decreased elution rate. The elution profile of kinetin with 1 *M* ammonia solution became a sharp peak (Fig. 1), yielding a 99% recovery.

HPLC conditions

Determination of kinetin was attempted using an ODS column. Ernstsen and Jensen [10] have investigated all binary systems of methanol or acetonitrile and water, and controlled the pH using an aqueous phase of either 20 m*M* ammonium acetate (pH 7.0) or 20 m*M* acetic acid (pH 3.5). On the other hand, Hardin and Stutte [11] used a 15-min linear gradient from 25% (in 0.67% acetic acid) to 32% methanol. Some of the major plant hormones were isolated by these methods. However, the separation of kinetin from *P. ginseng* callus extract was unsatisfactory. Adding trifluoroacetic acid to the solvent system of acetonitrile–water allowed a high resolution of kinetin and a linear calibration graph within the range 0.1–5 μg g^{-1} was obtained.

Recovery of kinetin

Table I shows recoveries of kinetin from dried callus, fresh callus and medium, spiked at 5, 1 and 0.5 μg g^{-1}, respectively. The recoveries were 72.0, 72.8 and 84.2%, respectively. The detection limit was 0.02 μg g^{-1}. Fig. 2 shows chromatograms of dried callus and spiked extracts.

Kinetin content in dried callus

The chromatogram of dried callus extracts is shown in Fig. 2(1). A peak corresponding to kinetin was not detected. The same extract was then analysed by HPLC with the DAD system (Fig. 3). A

TABLE I

RECOVERY OF KINETIN FROM DRIED CALLUS, FRESH CALLUS AND CULTURE MEDIA

Sample	Added ($\mu g\ g^{-1}$)	Recovery (%)	Mean ± S.D. (%)
Dried callus			
No. 1	5.0	73.7	
No. 2	5.0	74.5	72.0 ± 3.7
No. 3	5.0	67.8	
Fresh callus	1.0	72.8	
Medium	0.5	84.2	

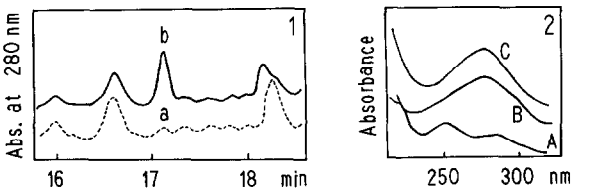

Fig. 3. (1) Magnified chromatograms of the extract from dried and spiked callus detected by DAD and (2) UV spectra. (1) The dashed line indicates chromatogram of the extract and the solid line indicates that of the spiked extract. The retention times of peaks a and b correspond to that of kinetin. (2) Lines A and B indicate the UV spectra of peaks a and b, respectively, and line C is that of a standard solution of kinetin detected by DAD.

small peak (a) was observed that corresponded to kinetin on magnification of the chromatogram [Fig. 3(1)]. Although peak a had a different spectrum to kinetin, peak b was identical [Fig. 3(2)]. The level of kinetin residues in dried callus was lower than the detection limit.

Uptake of kinetin from medium to callus

Pg-1 callus of *P. ginseng* was transplanted to fresh medium containing 0.1 $\mu g\ g^{-1}$ of kinetin and cultured as above. The callus and the medium were withdrawn after 0, 1, 2, 4 and 28 days and analysed for kinetin. As shown in Fig. 4, within 1 day the kinetin content in the callus reached a maximum, then decreased and after 28 days no kinetin was detected. The kinetin concentration in the medium decreased by 50% after 4 days, and it could not be detected after the 28th day. We found that ginseng callus absorbs kinetin from the medium rapidly and that the kinetin is metabolized it. Hence, kinctin was not detected in both callus and medium after the 28th day.

When *P. ginseng* was cultured by Furuya *et al.*'s method [12], it appeared that concentration of kinetin residue was below the detection limit. However, some products from tissue cultured plants may contain kinetin, depending on the concentration of kinetin in the growth medium.

Until recently, the toxicity of kinetin has been the subject of only a limited number of studies. Kajimoto *et al.* [14] showed that long-term administration of a small amount of kinetin resulted in weight in-

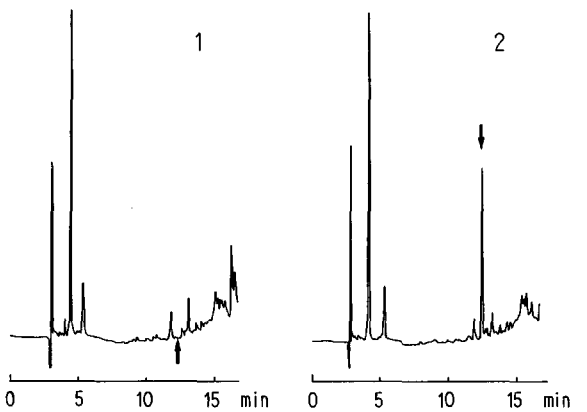

Fig. 2. Chromatograms of the extracts from (1) dried and (2) spiked callus of *P. ginseng*. Arrows indicate the spot corresponding to the kinetin peak.

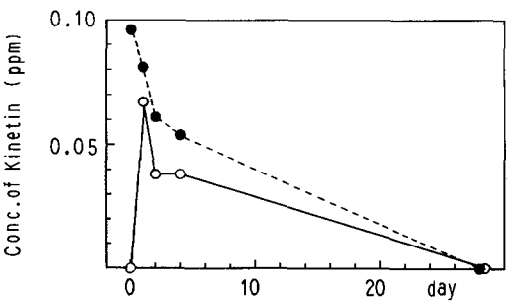

Fig. 4. Time course of uptake of kinetin from the medium by *P. ginseng* callus. Callus was transplanted to fresh medium supplemented with 0.1 $\mu g\ g^{-1}$ of kinetin and cultured for 28 days. Callus samples were withdrawn after 1, 2, 4 and 28 days and medium samples after 0, 1, 2, 4 and 28 days. ○ = Kinetin concentration in the callus and ● = that in the medium.

creases in dogs and mice, and that its LD_{50} value following intraperitoneal administration in the mous was 450 mg kg^{-1}. Farrow *et al.* [15] examined the effect of kinetin on the incorporation of uridine and thymidine into the nucleic acids of human leukocytes. Apparently the effects of kinetin on the treated animals were not serious, but the intake levels for humans need to be confirmed, since kinetin inhibits or stimulates nucleic acid synthesis in plant systems as well as in human leukocytes [15]. The method presented here will be applicable to the analysis of kinetin from other products of plant biotechnology.

ACKNOWLEDGEMENT

This research was supported by a grant from Japan Health Sciences Foundation.

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