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Batch immunoextraction method for efficient purification of aromatic cytokinins

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Abstract

A range of benzylaminopurines naturally occur in plants and exhibit high biological activity. Others have been synthesized, such as 6-(2-hydroxy-3-methoxybenzylamino)purine riboside (2OH3MeOBAPR), which has shown interesting anti-cancer activity under in vitro conditions. In order to study the biological activity of this interesting compound in more detail, a rapid and highly efficient method for its purification from complex samples (e.g. blood and plant extracts) is needed. Therefore, we prepared monoclonal antibodies against 2OH3MeOBAPR. The antibody had undetectable cross-reactivity with all natural isoprenoid cytokinins, but relatively high cross-reactivity with aromatic cytokinins as well as some synthetic di- and tri-substituted 6-benzylaminopurines and the corresponding ribosides. The antibody also showed strong responses and specificity in enzyme-linked immunoassays (ELISAs). In addition, it was used to prepare, for the first time, an immunoaffinity sorbent with high specificity and capacity for aromatic cytokinins. A batch immunoextraction method was then developed and optimized for the purification of 2OH3MeOBAPR from murine blood samples. The high efficacy and simplicity of this method (in off-line combination with HPLC-MS) for the isolation of target analytes from biological material is demonstrated in this study.
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1. Introduction

Cytokinins are an important group of plant growth regulatory substances [1,2]. They promote, in the presence of auxin, cell division in plant tissue cultures and affect a wide range of biological processes, including seed germination, bud differentiation, branching, chlorophyll and starch production, plant-pathogen resistance, apical dominance and leaf senescence. These compounds are derived from adenine, substituted at the N^6-position with an isoprenoid or aromatic side-chain. They mainly occur endogenously as free bases, nucleosides, glucosides and nucleotides, and are often present at very low concentrations (pmol g^{-1} fresh weight).

Moreover, some of the cytokinin-derived compounds are specific inhibitors of cyclin-dependent kinases [3] and exhibit interesting therapeutic effects against various types of diseases, especially cancer [4,5]. In our laboratory, more than 40 derivatives of 6-benzyladenosine, with various substituents on the benzyl ring, have been recently prepared, characterized and tested in different plant and animal systems [6]. To study the most promising compounds in more detail, a rapid and highly efficient method for purifying them from complex samples (e.g. blood and plant extracts) is urgently needed.

It is known that immunoaffinity purification methods, which are based on antibody-antigen interactions, can be highly specific for their respective target analytes [7,8]. This approach can provide selective sample enrichment, and thus greatly enhance detection limits of trace analyses. Off-line configurations for such methods include immunofiltration with non-immobilized antibodies or membrane strips with antibody (or antigen)-immobilized zones and immunoaffinity extraction using diverse types of immunosorbents. For immunoaffinity-based extractions, the immunosorbent is often packed into a disposable

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cartridge and the purification procedure consists of several distinct steps – conditioning, sample introduction, washing, elution of the target analytes and regeneration. An appropriate amount of the eluted extract is then injected into the selected separation system. This approach is very simple to perform and standard experimental equipment can be used [7,9]. However, in classic configurations, it can be quite time-consuming and inappropriate for automation. Therefore, an alternative approach to immuno-based sample preparation followed by either off-line or on-line coupling to various separation and/or detection systems has been developed [7,9]. New trends in environmental trace-analysis of organic pollutants with class-selective immunoextraction and clean-up in single steps using immunosorbents were presented in work by Pichon et al. [10]. The examples discussed in the cited paper, and other recent studies, have reported the coupling of immunosorbents in off-line or on-line solid phase extraction procedures with LC analysis and various detection modes. This is a powerful approach for the determination and quantification of diverse organic compounds that are present in minute quantities in various biological matrices, with no need for an additional clean-up step [11–15]. The term on-line immunoextraction [7,9,16] refers to the use of an immunoaffinity chromatography column to remove a specific solute or group of solutes from a sample prior to determination by an analytical method such as HPLC, GC, CE (most commonly HPLC, to date) coupled with MS. The immunoaffinity column serves as a sample clean-up step. The antibody is immobilized, either covalently or non-covalently, to the immunoaffinity column support material. In the on-line approach, a precolumn packed with the sorbent used for the extraction step is placed at the loop position of a six-port switching valve. By switching the valve, at appropriate times, non-retained components are flushed to waste and compounds trapped on the immunoaffinity sorbent are eluted on-line to the analytical column. High performance materials such as small rigid particles based on silica or synthetic polymers are used that can withstand the flow rates and pressure of the HPLC system. The on-line experimental set-ups are quite simple, automatable and they involve relatively little sample handling and solvent consumption. On-line immunoextraction techniques have been developed for a wide spectrum of analytes, such as phenylureas [17], benzidine, congeners and related azo dyes in surface water and industrial effluents [18], benzodiazepines [19], corticosteroids in urine [20], LSD and its analogues and metabolites in urine [21]. Tang et al. [16] have published a review summarizing the suitability of coupling immunoassays either pre- or post-column on-line with HPLC separation.

Cytokinin analysis requires very sensitive analytical tools, and HPLC/MS [22–30] is currently the most widely used approach for this purpose. The use of capillary electrophoresis [31–33] and GC/MS following derivatization of cytokinin-containing samples [34] has also been described. The cytokinin analysis needs to be preceded by quantitative extraction and purification of target substances from biological materials and their transfer into solutions with a high percentage of organic solvent by solid phase extraction [35,36] followed by immunoaffinity chromatography [8].

Strnad et al. [37,38] have developed polyclonal antibodies for the quantification and identification of both isoprenoid and aromatic cytokinins by ELISA. The same antigen preparation processes have been adopted to obtain monoclonal antibodies that could be used to prepare immunoaffinity sorbents. The utilization of conventional immuno clean-up methods for the isolation of cytokinins from various biological materials has also been described [23–25,34].

Immuno-based purification techniques can be used as sensitive and relatively cheap methods for purifying various types of samples. Here, we describe for the first time an off-line batch immunoaextraction method for aromatic cytokinins that is specific, rapid, simple, easy to use and cost-effective compared to conventional clean-up methods.

2. Experimental

2.1. Materials

Ovalbumin (OVA), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), Freund's adjuvant, Tris[hydroxymethyl] aminomethane (Tris), polyethylene glycol, formic acid, methanol, acetonitrile, immobilized protein G, ethanolamine. HCl and glycine were obtained from Sigma, alkaline phosphatase (3000 U mg^{-1}) from Roche Diagnostics, UltimaGold scintillation cocktail from Packard, and ammonium hydroxide from Merck. Lachema supplied sodium dihydrogen phosphate dihydrate, sodium chloride, sodium hydroxide, *p*-nitrophenylphosphate, sodium azide, magnesium chloride, sodium hydrogen-carbonate, ammonium sulfate, sodium borohydride, 3-(*N*-morpholino)propanesulfonic acid (MOPS). Affi-Gel 10 was purchased from Bio-Rad Labs. Milli-Q water was used throughout. All other solvents and chemicals used were of standard p.a. quality. 2OH3MeOBAPR and other cytokinins, di- and tri-substituted on the benzyl ring (Table 1), were synthesized as previously described [6,39]. Naturally occurring isoprenoid and aromatic cytokinins (Table 1) were a generous gift from Olchemim Ltd.

The following buffers were used: TBS (6.05 g Tris, 0.584 g NaCl, 0.203 g $MgCl_2$/L, 0.2% NaN_3, pH 7.5), PBS (7.8 g $NaH_2PO_4 \cdot 2H_2O$, 0.877 g NaCl/L, pH 7.2 or 7.4), and borate buffer (3.81 g $Na_2B_4O_7 \cdot 10H_2O$, 0.58 g NaCl, 0.20 g $MgCl_2$/L, pH 9.5).

2.2. Preparation of antigens

The 2OH3MeOBAPR-protein conjugate was prepared by a modification of the periodate oxidation method [40], briefly as follows. 2OH3MeOBAPR was dissolved in 10% aqueous DMSO at a concentration of 10 mg/mL and 1 mL of 50 mM $NaIO_4$ solution was added dropwise. The solution was stirred for 10 min in the dark at room temperature, excess periodate was decomposed by the addition of 42 μL of 5% aqueous ethylene glycol and the mixture was shaken for another 5 min.

BSA and OVA were dissolved in 0.01 M borate buffer at a concentration of 15 mg/mL. The activated hapten solution was added in 50 μL aliquots to the protein solutions. The reaction

Table 1
Abbreviations of used compounds

Compound	Abbreviations
6-Benzylaminopurine	BAP
Dihydrozeatin	DHZ
Isopentenyladenine	iP
cis-Zeatin	*c*Z
trans-Zeatin	*t*Z
meta-Topolin	*m*T
ortho-Topolin	*o*T
para-Topolin	*p*T
6-(3-Methoxybenzylamino)purine	Me*m*T
6-(2-Methoxybenzylamino)purine	Me*o*T
6-(4-Methoxybenzylamino)purine	Me*p*T
Kinetin	K
6-(3,5-Dihydroxybenzylamino)purine	3,5diOHBAP
6-(2-Hydroxy-3-methoxybenzylamino)purine	2OH3MeOBAP
6-(3,5-Dimethoxybenzylamino)purine	3,5diMeOBAP
6-(3-Hydroxy-4-dimethoxybenzylamino)purine	3OH4MeOBAP
6-(2,3-Dimethoxybenzylamino)purine	2,3diMeOBAP
6-(2,4-Dimethoxybenzylamino)purine	2,4diMeOBAP
6-(3,4-Dimethoxybenzylamino)purine	3,4diMeOBAP
6-(2,4,6-Trimethoxybenzylamino)purine	2,4,6triMeOBAP
6-(3,4,5-Trimethoxybenzylamino)purine	3,4,5triMeOBAP
6-(4-Hydroxy-3,5-dimethoxybenzylamino)purine	4OH3,5diMeOBAP

R in each compound represents 9-β-D-ribofuranoside and 9G means 9-β-D-glucopyranoside.

mixtures were then stirred at room temperature for 1 h. In order to stabilize the conjugate, 7.5 mg $NaBH_4$ was added to both solutions and the mixtures were stirred for another 1 h. Finally, the conjugates were dialyzed against PBS buffer (pH 7.4) for 5 days. The coupling ratio was determined to be 16 mol 2OH3MeOBAPR/mol BSA from the UV spectra of the conjugates (λ_{max} = 270.2 nm).

The same reaction was used to synthesize 2OH3MeOBAPR – alkaline phosphatase (AP) conjugates using a reagent ratio of 5:1.

2.3. Immunization schedule and preparation of monoclonal antibodies

Six-week-old female BALB/c mice were immunized intraperitoneally with 50 μg/0.4 mL PBS (pH 7.2) of the 2OH3MeOBAPR-BSA conjugate emulsified with an equal volume of complete Freund's adjuvant. Booster injections of a further 50 μg were given in incomplete Freund's adjuvants at 2-week intervals. Three additional subcutaneous injections (30 μg/0.4 mL PBS) were given 3–1 days prior to the fusion of spleen cells.

The fusion and subsequent hybridoma selection was performed as described by Harlow and Lane [41]. Hybridomas designated mAb8/1–mAb8/6, secreting anti-2OH3MeOBAPR antibodies were detected by a DOT-BLOT technique using 2OH3MeOBAPR-OVA conjugate, cloned by limiting dilution and stored in liquid nitrogen or injected into BALB/c mice to induce ascitic tumor formation.

For antibody preparation, 6-week-old BALB/c mice were injected intraperitoneally with 0.5 mL of incomplete Freund's adjuvant. Animals were transplanted with the selected

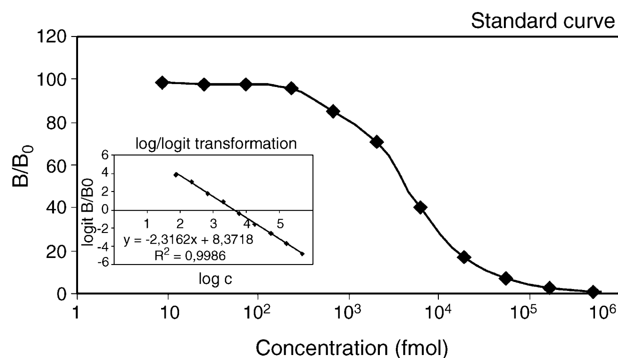

Fig. 1. Sigmoidal standard curves for 2OH3MeOBAPR and (inset) curve linearized by logit–log transformation.

hybridoma cell line (7 × 10^5 cells in 0.5 mL of PBS) 10 days later intraperitoneally. Ascitic fluids were typically collected after 8 days. The mAbs were purified on a protein G column and precipitated with $(NH_4)_2SO_4$ (40%, v/v). The precipitate containing antibodies was re-dissolved in PBS and dialyzed against PBS with 5% $(NH_4)_2SO_4$ (pH 7.5) for 24 h, PBS (pH 7.5) for 24 h, and finally against 0.1 M MOPS buffer containing 0.3 M NaCl, pH 7.2 for 48 h at 4 °C.

2.4. Monoclonal antibody characterization

The mAb subclasses were determined by the double diffusion method of Ouchterlony [42]. The mAb8/1–mAb8/6 antibodies were further characterized by enzyme-linked immunosorbent assay (ELISA) (Table 2), using a modification of the ELISA protocol described by Strnad [38]. Ninety-six-well microtiter plates (Gamedium, Czech Republic) were coated with 150 μL of mice anti-2OH3MeOBAPR antibodies (0.25 mg/mL in 50 mM $NaHCO_3$, pH 9.6), incubated overnight at 4 °C for binding, and then washed twice with redistilled H_2O to remove unbound antibody. The wells were subsequently filled with 200 μL 0.02% BSA in TBS and incubated for 1 h at 25 °C. After decanting and two washes with redistilled H_2O, the wells were filled with 50 μL TBS, 50 μL of standards or samples in TBS and 50 μL of 2OH3MeOBAPR-AP tracer in 0.02% BSA in the stated sequence. The plates were then incubated at 25 °C for 1 h. The unbound AP conjugate was removed by rinsing the plates four times with dist. H_2O and the wells were then filled with 150 μL of *p*-nitrophenylphosphate as a substrate (1 mg/mL 50 mM $NaHCO_3$, pH 9.6). The reaction was stopped after 1 h by adding 50 μL 0.5 M NaOH and the absorbance was measured at 405 nm in a Titertek Multiscan® PLUS ELISA reader (Labsystems, Finland). Sigmoidal curves for standards and cross-reacting compounds were linearized by log–logit transformation [43] as follows: logit $B/B_0 = \ln\{(B/B_0)/(100 - B/B_0)\}$, where B and B_0 represent binding of the alkaline phosphatase tracer in the presence and absence of standard (data for mAB8/1 are shown in Fig. 1).

The specificity of the resulting monoclonal antibodies was determined by cross-reactivity [7] analyses. For the competition experiments 2OH3MeOBAPR standard was replaced either by a naturally occurring cytokinin (Table 3) or by a 2OH3MeOBAPR synthetic analogue (Table 4).

Table 2
Characteristics of monoclonal antibodies mAb8/1–mAb8/6 in ELISA

Characteristics	8/1	8/2	8/3	8/4	8/5	8/6
Concentration of antibody (mg/mL)	9.4	9.2	10.2	10.5	10.9	9.4
Detection limit of assay (mol/L)	1.34×10^{-8}	1.4×10^{-8}	1.38×10^{-8}	1.4×10^{-8}	4.0×10^{-8}	1.4×10^{-8}
Midrange B/B_o (50% binding) (mol/L)	8.2×10^{-8}	8.4×10^{-8}	1.02×10^{-7}	8.08×10^{-8}	1.1×10^{-7}	7.8×10^{-8}
Range of measurement (mol/L)	1.34×10^{-8} to 3.8×10^{-7}	1.4×10^{-8} to 3.6×10^{-7}	1.38×10^{-8} to 3.8×10^{-7}	1.4×10^{-8} to 3.7×10^{-7}	4×10^{-8} to 3.6×10^{-7}	1.4×10^{-8} to 3.8×10^{-7}
Non-specific binding (%)	1.08	2.9	3.8	0.45	2.9	2.8
Intraassay variability (%)	0.80	1.45	1.02	2.4	1.78	0.87

Table 3
The cross reactivity (%) of six hybridomas mAb8/1–mAb8/6 with naturally occurring cytokinins

Compound	8/1	8/2	8/3	8/4	8/5	8/6
iP	<0.1	<0.1	<0.1	0.1	<0.1	<0.1
iPR	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
BAP	0.2	0.2	0.2	0.1	0.2	0.1
BAPR	0.2	0.2	0.2	0.1	0.2	0.2
*o*T	0.3	0.3	0.3	0.3	0.3	0.3
*o*TR	1.3	2.4	2.5	2.3	2.4	2.3
*o*T9G	1.2	1.7	1.2	1.2	1.2	0.7
*p*T	0.7	1.0	1.1	1.0	1.1	1.3
*p*TR	0.9	0.7	1.0	5.4	0.9	0.9
*m*T	1.2	1.1	1.4	6.7	1.2	1.1
*m*TR	1.1	1.0	1.1	1.0	1.0	1.0
*m*T9G	1.5	1.9	1.5	1.6	1.7	0.9
Me*o*T	0.4	0.6	0.9	4.9	0.8	0.5
Me*o*TR	1.8	2.2	2.2	2.1	2.0	2.1
Me*m*T	2.7	2.5	2.9	3.0	2.7	2.8
Me*m*TR	3.8	4.0	4.6	4.6	4.3	4.7
Me*p*T	13.3	15.3	15.5	13.2	15.9	13.9

Only the compound with cross reactivity higher than 0.01% are included.

2.5. Preparation of immunoaffinity gel and development of the batch immunoextraction method

The purified antibody mAb8/1 was mixed with Affi-Gel 10 (20 mg of antibody/mL of the gel) and the suspension was gently shaken at 4 °C for 4 h. As a precaution, any remaining active ester was blocked by adding 0.1 mL ethanolamine. HCl/mL gel (pH 8.0) and mixing for 1 h at 4 °C. After transferring the immunoaffinity gel (IAG) to a polypropylene column (6 mL, 20 μm, Supelco), the unbound antibody was washed out with PBS (pH 7.4) until the absorbance (A_{280}) of the eluate had fallen to baseline levels.

The capacity of mAb 8/1-based IAG was tested by adding 10 μL portions of it to standard solutions containing amounts of 2OH3MeOBAPR ranging from 5 to 1000 pmol in 5% EtOH/PBS buffer (v/v) in Eppendorf tubes.

The IAG was further characterised using two different cytokinin mixtures. The first (Mixture I) contained 24 natural isoprenoid and aromatic cytokinin standards – including bases, ribosides and 9-glucosides (listed in table 5) – in 70% ethanol. The second mixture (Mixture II) contained 14 synthetic aromatic cytokinin derivatives, including bases and ribosides (Table 5), in 70% ethanol. Solutions containing equal amounts (ranging from 5 to 200 pmol) of each tested standard in 5% EtOH/PBS buffer (v/v), were prepared in Eppendorf tubes and mixed with 10 μL IAG.

Solutions at each concentration were prepared in triplicate and the whole experiment was repeated twice. The tubes were stirred for 2 h at laboratory temperature. The samples were subsequently filtered in SPE tubes (1 mL, 20 μm frit, Supelco) that had been pre-rinsed sequentially with 1 mL MeOH, 1 mL

Table 4
The cross reactivity (%) of six hybridomas mAb8/1–mAb8/6 with new synthetic di- and tri-substituted aromatic cytokinin derivatives

Compound	8/1	8/2	8/3	8/4	8/5	8/6
2OH3MeOBAPR	100	100	100	100	100	100
2OH3MeOBAP	22.6	26.9	27.7	34.0	65.2	27.1
3,5diMeOBAPR	0.7	0.7	0.7	0.7	2.6	0.8
3,5diMeOBAP	0.3	0.3	0.3	0.4	1.3	0.5
3OH4MeOBAPR	1.9	3.8	4.9	3.3	3.0	4.0
3OH4MeOBAP	0.8	1.0	1.0	0.9	1.1	1.4
2,3diMeOBAPR	414.2	1140.2	571.3	906.0	1080.1	1310.4
2,3diMeOBAP	47.4	103.2	148.0	152.8	107.6	151.6
2,4diMeOBAPR	344.8	314.6	382.7	489.2	773.1	576.3
2,4diMeOBAP	81.4	129.6	117.1	139.4	241.8	191.0
3,5diOHBAPR	2.4	1.9	1.9	1.8	2.9	4.1
4OH3,5diMeOBAPR	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
4OH3,5diMeOBAP	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
3,4diMeOBAPR	10.0	4.9	10.3	10.3	9.2	9.8
3,4diMeOBAP	5.1	3.6	5.1	4.3	4.2	4.5
2,4,6triMeOBAP	233.0	260.0	243.0	1840.0	313.0	476.0
3,4,5triMeOBAP	0.2	0.1	0.2	0.2	0.4	0.4

Table 5
Retention times and dwell times of all analyzed compounds in mixtures I and II

Compound in mixture I	m/z	Retention time (min)	Dwell time (s)
Z9G	382.06	11.32	0.94
DHZ9G	384.09	12.14	0.94
tZ	220.08	13.66	0.63
cZ	220.08	15.19	0.63
dHZ	222.09	14.67	0.63
mT9G	404.03	15.17	0.63
K	216.05	18.62	0.47
pT	242.03	16.82	0.47
mT	242.03	18.35	0.47
tZR	352.07	16.84	0.47
cZR	352.07	17.93	0.47
dHZR	354.10	17.78	0.47
oT	242.03	23.70	0.31
KR	348.03	20.74	0.31
iP9G	366.08	22.07	0.31
pTR	374.05	19.35	0.31
mTR	374.05	20.48	0.31
BAP9G	388.07	21.01	0.31
oT9G	404.04	19.48	0.31
iP	204.10	26.67	0.38
BAP	226.06	25.63	0.38
iPR	336.08	27.80	0.38
BAPR	358.06	26.67	0.38
oTR	374.05	24.77	0.38
Compound in mixture II	**m/z**	**Retention time (min)**	**Dwell time (s)**
3,5diOHBAPR	390.02	2.97	0.70
3,4diMeOBAP	286.05	4.94	0.70
2OH3MeOBAP	272.03	5.52	0.70
3OH4MeOBAPR	404.04	7.45	0.70
2,3diMeOBAP	286.05	7.74	0.70
MemT	256.03	8.47	0.47
2,4,6triMeOBAP	316.06	9.16	0.47
2,4diMeOBAP	286.05	9.36	0.47
3,4diMeOBAPR	418.07	12.62	0.47
2OH3MeOBAPR	404.07	12.87	0.47
2,3diMeOBAPR	418.07	15.99	1.41
MemTR	388.06	16.23	1.41
MeoTR	388.06	16.85	1.41
2,4diMeOBAPR	418.07	17.09	1.41

H_2O and 1 mL PBS. The analyte-containing gel was washed with water and eluted by 2 mL of 0.2% HCOOH in MeOH at −20 °C. Used gel was collected in a polypropylene column and regenerated with a cycle of 1 mL portions of PBS–H_2O–0.2% HCOOH/MeOH (−20 °C)–H_2O–PBS. Regenerated IAG can be repeatedly used, so the collected gel was stored at 4 °C in PBS containing 0.2% NaN_3.

After elution, the samples were evaporated to dryness in a Speed-Vac concentrator (UniEquip) and analyzed using a high performance liquid chromatography (HPLC) system coupled to an ultraviolet-diode array detector (UV-DAD) and a mass detector (MS), as described below.

2.6. Chromatography and mass spectrometry conditions

All standard samples were analyzed by high performance liquid chromatography (Waters; model Alliance 2690) linked to a Micromass ZMD mass spectrometer equipped with an electrospray interface (LC (+)ESI-MS) and photodiode array detector (UV-DAD) (Waters; model 996) (1:1 post-column splitting). HPLC conditions were as follows: flow rate, 0.25 mL/min; column temperature, 30 °C; sample heater temperature, 4 °C. UV-DAD data were acquired between 210 and 400 nm. MS analyses were carried out under the following conditions: source block temperature, 100 °C; desolvation temperature, 250 °C; capillary voltage, +3.0 kV; cone voltage, 20 V. Nitrogen was used as both desolvation (500 L/h) and cone gas (50 L/h). Under these conditions, quantitative analysis was performed in selective ion recording (SIR) mode with dwell time 0.94 s. All data acquired were processed by MassLynx software.

The purified standard samples were dissolved in 60 μL MeOH/H_2O (30/70) and 30 μL of each sample was injected onto a C18 reversed-phase column (Waters; Symmetry; 3.5 μm; 150 mm × 2.1 mm). The mobile phase consisted of gradients of MeOH (solvent A) and 0.1% ammonium formate buffer pH 2.9 (solvent B for the 2OH3MeOBAPR standard samples and Mixture II), and 15 mM ammonium formate buffer pH 4.0 (solvent B for Mixture I). For the 2OH3MeOBAPR standard samples the column was eluted with a linear gradient of 30–80% A (0–10 min), followed by isocratic elution with 80% A (10–12 min). Under these conditions the retention time of 2OH3MeOBAPR was 8.91 min, when monitored by the photodiode array detector ($\lambda_{max} = 268$ nm), and 8.64 min when monitored by the MS detector $\{[M+H]^+ = 403.94\}$. Mixture I was eluted from the column by a linear gradient of 10–50% A from 0 to 25 min, followed by isocratic elution at 50% A (25–28 min), while Mixture II was eluted using the following program: 0–5 min 30% A, 5–10 min 30–40% A, 10–12 min 40–50% A, 12–20 min 50% A. Retention times, *m/z* and dwell times of all analyzed compounds are listed in Table 5.

2.7. Preparation of [^{3}H] 6-(3-methoxybenzylamino)purine-9-β-D-ribofuranoside

6-(3-Methoxybenzylamino)purine-9-β-D-ribofuranoside (MemTR) (2.8 μmol, 1.1 mg) and 3.8 mg 10% $PdO/BaSO_4$ were dissolved in 0.2 mL of a mixture of dioxane and 0.1 M Na_2CO_3 (2:1, v/v). The reaction mixture was stirred for 1 h in a 40% tritium atmosphere at 0.075 MPa. After removal of the labile radioactivity (by lyophilization of the crude product, solution in 9:1 *tert*-butanol:water, separation of the catalyst by centrifugation and another lyophilization step), the purity of the crude product was 92% (according to TLC analysis on a silica gel with fluorescent indicator, Aldrich, in $CHCl_3$:MeOH:HCOOH, 80:20:2, v/v/v, $R_F = 0.62$). It was further purified by preparative TLC under the same conditions. The total yield of [^{3}H]MemTR was 520 MBq (14.1 mCi) with radiochemical purity higher than 98% and specific activity of 285 GBq mmol^{-1} (7.7 Ci mmol^{-1}).

2.8. Testing of recovery of batch IAE using [^{3}H]MemTR

Blank murine serum (100 μL) and 10 μL of [^{3}H] MemTR standard in acetonitrile (50.000 dpm) were mixed and 40 μL of acetonitrile was added to each sample to precipitate proteins.

After centrifugation (4800 × *g* for 10 min at 4 °C), supernatants were purified by solid phase extraction on 360 mg Sep-Pak C18 cartridges (Waters) activated with acetonitrile. After the evaporation, samples were purified by batch IAE. Purified samples were evaporated to dryness, dissolved in MeOH/H_2O (30/70) and analyzed by HPLC/UV (under the conditions described for Mixture II in Section 2.6). The peak corresponding to MemTR, retention time 16.18 min, was collected. The radioactivity of the collected fractions, prepared in triplicate, was measured by an LS 6500 multi-purpose scintillation counter (Beckman, USA).

2.9. Preparation, treatment and analysis of murine blood samples

Six- to eight-week-old female Balb/c mice were treated orally with a single 10 mg dose of 2OH3MeOBAPR or vehicle. The drug was suspended in 250 μL of 1% methylcellulose and applied through gastric metallic catheters to five animals per group. Animals were sacrificed 2 h later by exsanguination from brachial plexus vessels under ether anesthesia. Collected blood was kept on ice and serum was separated within 60 min of sampling at 3000 × *g* for 10 min at 4 °C and stored at −80 °C for further analyses.

Each serum sample was divided into two aliquots (A and B) and acetonitrile was added to each aliquot (3 mL/0.5 mL serum) to precipitate proteins, which were then removed by centrifugation at 4800 × *g* for 10 min at 4 °C. Supernatants were purified by solid phase extraction on Sep-Pak C18 cartridges (Waters) activated with acetonitrile. After evaporation, aliquot A was used for direct LC (+)ESI-MS measurements while aliquot B was purified by batch immunoextraction before the analysis.

Blood samples were analyzed under the same conditions as standard 2OH3MeOBAPR samples, in both selected ion recording (SIR) mode and full scan ion recording LC (+)ESI-MS mode in the range from 50 to 500 *m*/*z* (to confirm the peak identities). The purified samples were dissolved in MeOH/H_2O (30:70), filtered through a PTFE microfilter (4 mm, 0.45 μm, Waters) and 30 μL of each sample (50% of the total sample volume before filtration) was injected onto a C18 reversed-phase column. 2OH3MeOBAPR levels in murine blood samples were determined using an external calibration curve.

3. Results and discussion

3.1. Antibody preparation and characterization

Spleen cells, derived from a mice immunized with the 2OH3MeOBAPR-BSA conjugate, were fused with SP2 myeloma cells and hybridomas, which excreted anti-2OH3MeOBAPR antibodies, were isolated. After cloning and subsequent selection of cells using an ELISA, six cell lines (mAb8/1–mAb8/6), excreting mAbs showing high binding capacity either to 2OH3MeOBAPR-OVA (Dot-Blot) or -AP tracer conjugate, were selected. These six mAbs were analyzed in more detail and their basic characteristics are presented in Table 2. As observed in several previous studies of anti-cytokinin antibodies [43–46] our mAbs showed some variations in antibody class, the affinities of the mAb for 2OH3MeOBAPR and their detection ranges in ELISA assays. The antibody with the most promising characteristics appeared to be mAb8/1, so it was used in all further studies. This antibody is of the IgG_1 subclass as determined by the Outcherlony immunodiffusion assay (data not shown). A standard curve for the mAb8/1-based ELISA is given in Fig. 1. The inset shows the linearized curve, providing a measuring range between 1.38×10^{-8} mol/L and 3.8×10^{-7} mol/L. The assay was very sensitive since as little as 1.34×10^{-8} mol/L of 2OH3MeOBAPR could be detected, giving comparable detection limits to other cytokinin ELISAs [47–50]. The mid-range values (amount of antigen required for 50% inhibition) were around 8.2×10^{-8} mol/L for this cytokinin-derived compound. Unspecific binding in the presence of an excess of standard (200 pmol) accounted for <3% of total binding. Within the measured range, the standard curves were almost linear over three orders of magnitude with small inter- and intra-assay variations (<3%).

The specificity of the antibodies was determined by cross-reactivity studies, and the results are shown in Tables 3 and 4. The natural and synthetic cytokinins were tested for antibody binding over a range from 0.01 to 5000 pmol/assay. Data for cytokinins and related compounds producing molar cross-reactivities lower than 0.01%, deemed to represent no cross-reactivity, are not shown in the tables. These compounds included adenine, adenosine, adenosine 5′-monophosphate, inosine, *N*,*N*'-diphenylurea, all metabolites of *trans*- and *cis*-zeatin, dihydrozeatin and isopentenyladenine, even when tested in amounts up to 5 nmol per assay. The natural isoprenoid cytokinins showed at most only slight cross-reactivity (only iP and iPR generated responses stronger than the 0.01% threshold; 0.09 and 0.04%, respectively). The cross-reactivity data suggest that the antibodies are strongly specific solely for aromatic cytokinins (adenine derivatives bearing an aromatic ring at the N^6-position).

The recognition properties of the mAbs are very unusual because of their highly generic antibody specificity for aromatic cytokinins. Apart from 6-(4-hydroxy-3,5-dimethoxybenzyl)aminopurine (4OH3,5diMeOBAP) and its riboside, which showed low cross-reactivities, all other natural and artificial aromatic cytokinins, as well as derived compounds used in the study, reacted significantly with some of the six mABs. The most reactive were 2,3-, 2,4-diMeO- and 2,4,6-triMeOBAPR derivatives, their reactivity exceeding that of the 2OH3MeOBAPR used in the immunogen. Surprisingly, topolins (monosubstituted BAPs: *m*T, *o*T, *p*T, their methoxy derivatives and ribosides) also showed very high levels of competition, especially *p*-methoxytopolin riboside. In addition to the ribosides, the antibodies cross-reacted strongly with the respective free bases (cross reactivity between 10 and 60%), riboside 5′-monophosphates, and 9-glucosides (data not shown). Low specificities for compounds with any substituent at N9 were consistently found when the cytokinin hapten and protein were conjugated via a spacer such as ribose [37]. In conclusion, this is the first report describing generic monoclonal antibodies for aromatic cytokinins. Our antibodies are highly specific for the N^6-aromatic substituent and do not cross-react with any natural isoprenoid cytokinin. This strong discrimination was found with all six mAbs and, hence, is presumably

typical of this kind of antibody. Nevertheless, the selectivity and high sensitivity of the ELISA described here suggest that 2OH3MeOBAPR provides a very good epitope for the recognition of aromatic cytokinins.

3.2. Immunoextraction characteristics

Immunoaffinity chromatography (IAC) is often used as a final purification step, which is usually essential for samples of complex biological matrices (blood, plant material, etc.) to be used for trace analyses. Much higher selectivity can be achieved by this method, compared with conventional solid-phase extraction (SPE) procedures, where co-extraction of analytes and matrix interferences is a common problem [7]. On the other hand, IAC in classical configurations (when using IAC columns) can be considered a low throughput method; relatively simple, but time consuming, laborious and unsuitable for automation. One possibility, which has the advantage of decreasing the amount of antibody required (and thus sample volumes and extraction times), is coupling immunoextraction on-line with LC [7]. Another possible way to perform rapid immunoextraction and decrease the amount of antibody needed per sample is to use IAC in a batch configuration.

In this paper we therefore describe the development of an immunoaffinity sorbent (IAG) that is highly specific for aromatic cytokinins, based on a generic monoclonal anti-2OH3MeOBAPR antibody. Due to the cross-reactivity of the monoclonal mAb8/1 antibody, the ability of the immunoaffinity sorbent, prepared by coupling the antibody to Affi-Gel 10, to trap 2OH3MeOBAPR, aromatic versus isoprenoid cytokinins and/or mixtures of synthetic BAP analogues was examined.

The bound phytohormones can be eluted with a variety of disruptive agents, but the most convenient for ARCK purification appears to be methanol. The immobilized mAbs show unexpected stability in this solvent, although there have been indications that some loss in initial capacity occurs with repeated use [51]. However, no such losses were observed in our study, since mAb8/1 IAG retained almost constant properties for at least 10 successive cycles.

An important immunoextraction parameter is capacity because the recoveries are no longer constant and the calibration range no longer linear if the IAG is overloaded. The sorbent capacity [7,9] is defined as the maximum amount of the analyte that can be adsorbed by a given volume of immunosorbent. In the immunosorption of small molecules, the binding efficiency and capacity depend mainly on the density of the antibody immobilized on the support and the volume of the IAG. Therefore, antibody concentrations recommended by the manufacturer (Bio-Rad Labs.) or reported by other authors (20 mg mL^{-1} gel) were used [52] in this study. Recovery of prepared immunosorbent, tested using [^{3}H]MemTR (since no tritiated standard of 2OH3MeOBAPR was available), was $39.0 \pm 2.1\%$.

When testing the dynamic capacity of our immunosorbent, amounts of 2OH3MeOBAPR standard ranging from 5 to 1000 pmol were applied to 10 μL of IAG, and the binding and recovery capacities were determined (Table 6). The IAG capacity was estimated to be about 14–17 nmol mL^{-1} of gel. Recoveries higher than 90% were obtained for 2OH3MeOBAPR in the range 5–50 pmol. With up to 200 pmol of the standard recoveries were still higher than 50% (Table 6). Beyond this limit, the immunoextraction recovery declined rapidly. There are several other reports in the literature concerning the use of anti-phytohormone antibodies for immunoaffinity chromatography [51–54]. Antibodies raised against a number of cytokinins have also been coupled to a variety of supports [55] to produce immunoaffinity matrices with capacities between 100 ng and 2 mg/mL. The capacity per mL of our gel, expressed according to Davis et al. [52], is similar to values published in other studies.

For IAC of cytokinins from plant extracts to be useful, when several cross-reacting metabolites are present, the capacity and recoveries must remain high for each of the target metabolites. The cross-reactivity parameters for a complex mixture may differ from those of the primary antigen for a number of reasons, e.g. (1) differences in the K_a values of antibodies for cross-reacting substance in comparison to the primary antigen, (2) differences in the behavior of immobilized antibodies on a gel and (3) substantial differences in the concentrations (relative and absolute) of individual cross-reacting substances in real samples [56]. Since our cross-reactivity studies only investigated the antibody behavior in the presence of a single free competitor, the batch immunoextraction-LC (+)ESI-MS method was used to determine the extent of cross-reactivity when immobilized antibody was exposed to mixtures of cytokinins. For this purpose, different amounts of a mixture containing equal concentrations (ranging from 5 to 100 pmol) of each of 24 isoprenoid and aromatic cytokinin standards (bases, ribosides and 9-glucosides; Mixture I, Fig. 2B) were mixed with 10 μL IAG and eluates were analyzed by LC (+)ESI-MS as described in Section 2.6. Fig. 3 provides a qualitative reference standard for the analyzed cytokinin mixture. Virtually no retention of isoprenoid cytokinins was observed, but the natural aromatic cytokinins (e.g. topolin ribosides) remained temporarily coupled. Cytokinins with no affinity to the sorbent included all zeatin, dihydrozeatin and isopentenyladenine derivatives. The IAG showed little, if any, binding of isopentenyladenosine, although MS detected trace amounts in the eluate (data not shown). In summary, the IAG mAb8/1 is highly specific for aromatic cytokinins, confirming the results of the cross-reactivity study.

To test the capacity of the IAG to bind different aromatic cytokinins, and the effects of competition between ARCKs with different relative affinities on those capacities, a mixture of ARCKs (Mix II) was applied to the IAG and their recoveries were determined. Fig. 2A shows the results of an experiment in which varying loads of equimolar amounts of 14 different ARCKs were applied. Three ligands, including 2,3diMeBAPR, its free base and 2,4diMeBAPR, were retained with high recoveries over the concentration range tested. The other seven showed concentration-dependent losses. The methoxytopolins and 3,5diOHBAPR showed the largest losses, and thus the lowest relative affinity. Binding sites for these cytokinins were almost completely saturated at loads of just 15–50 pmol (Fig. 2A and B). The results of these experiments indicate that the maximum capacity of the IAG for different cytokinins, and

Table 6
The test of capacity of mAb8/1 antibody with 10 μL of immunoaffinity gel for 5–1000 pmol of 2OH3MeOBAPR

Amount of substance in batch (pmol)	5	25	50	100	200	500	700	1000
Gel capacity (pmol/10 μl)	5.3	24.4	44.7	79.5	111.6	146.3	166.8	156.7
Recovery (%)	105.2	97.5	89.4	79.5	55.8	29.3	23.8	15.7
SD (%)	0.7	3.6	2.0	2.3	1.5	1.7	0.8	0.4

Each concentration was prepared in triplicate and the whole experiment repeated twice.

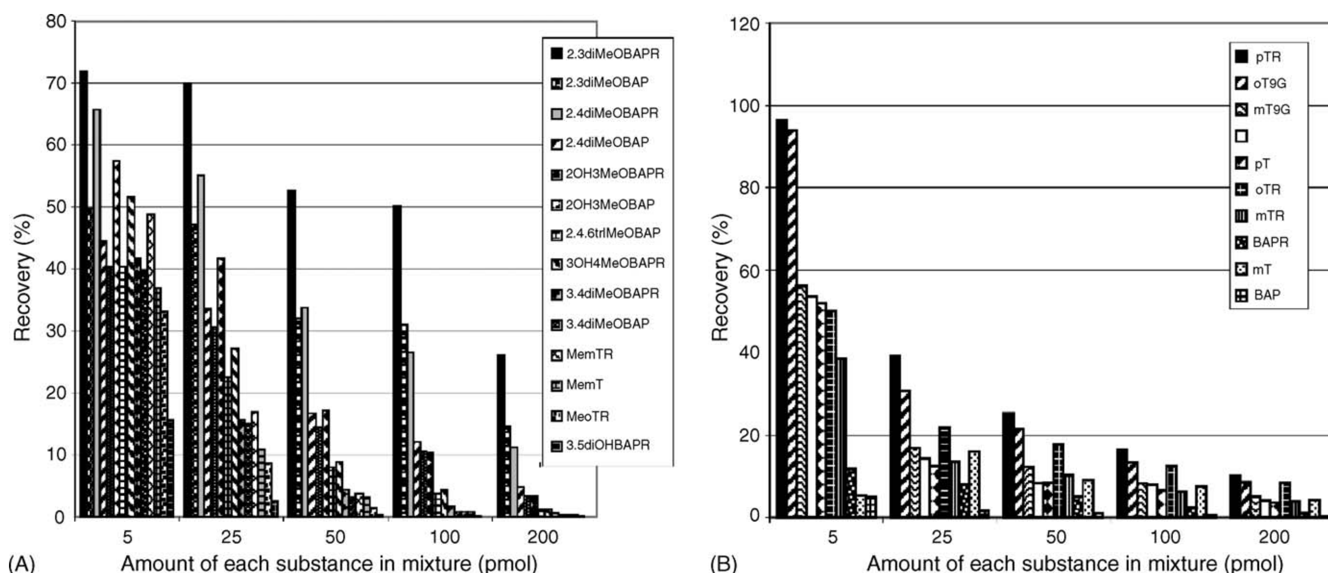

Fig. 2. Results of a test of binding capability of mAb8/1 antibody with 10 μL of immunoaffinity gel for a mixture of 14 synthetic di- and tri-substituted aromatic cytokinins (A) and a mixture of 24 naturally occurring cytokinin derivatives (B). Only compounds recovered in amounts greater than their respective limits of detection are included.

their recovery, differed depending on other ligands present in the natural samples. At all concentrations tested, the differences in capacities for individual cytokinins were parallel to the cross-reactivity obtained in the ELISAs and did not seem to be influenced by immobilization of the antibody used. They were however influenced by the remaining capacity of free antibody binding sites at the low concentrations tested. Furthermore, the total dynamic capacity of the gel for ARCK mixtures was comparable to the 2OH3MeOBAPR binding capacity. Finally, the antibody/gel ratio was considered to have high potential utility for the co-purification of cytokinins, since the total amount of cytokinins in non-transgenic plant material is always below 15–25 pmol g^{-1} fresh weight.

3.3. Analyses of 2OH3MeOBAPR in murine blood samples

The effectiveness of the batch immunoextraction method for purifying 2OH3MeOBAPR from blood samples in comparison to non-specific purification with C_{18} Sep-Paks was assessed using an LC system coupled to a diode array detector. Our results show that a large number of UV-absorbing substances were present after C18 solid phase extraction of deproteinized mouse

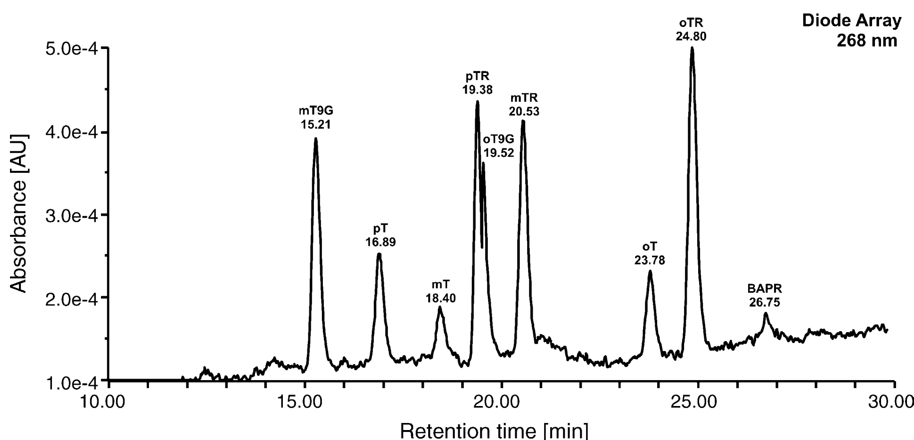

Fig. 3. HPLC/UV-DAD chromatogram (UV 268 nm) of an immunopurified sample containing a mixture of equal amounts of 24 isoprenoid and aromatic cytokinins.

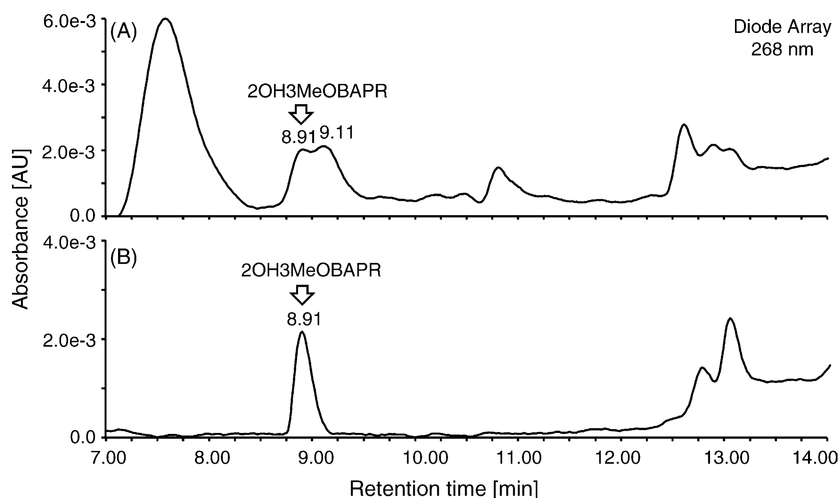

Fig. 4. HPLC/UV-DAD chromatograms (UV 268 nm) of murine blood samples (A: purified by Sep-Pak C18, B: purified by Sep-Pak C18 and batch immunoextraction with 10 µl of IAG).

sera. However, comparison of HPLC chromatograms before and after IAC (Fig. 4, data not shown for other matrices) shows that the IAC removed potentially interfering substances considerably more efficiently. The absence of interfering peaks in the vicinity of the peak corresponding to 2OH3MeOBAPR even allowed its quantification by UV peak area measurement. The comparison of LC-ESI(+)-MS chromatograms also clearly showed that the immunoaffinity purification led to an increase in the selectivity and sensitivity of the subsequent HPLC-MS analysis (data not shown).

HPLC-ESI(+)-MS quantification of 2OH3MeOBAPR in murine blood samples was performed using an external calibration curve. Linearity $y = 1.2678x + 3.1822$ ($R^2 = 0.9996$) for the concentration range 0.05–50 pmol was obtained in SIR mode. The limit of detection (LOD), defined as the lowest detectable concentration (S/N ≥3 peak-to-peak), was found to be 0.1 pmol of 2OH3MeOBAPR, and the lower limit of quantification, defined as the lowest measurable concentration (S/N ≥10 peak-to-peak), was 0.5 pmol of 2OH3MeOBAPR. The average serum concentration of 2OH3MeOBAPR was 0.27 µmol/L 2 h after administration of a single 10 mg dose of the substance with a standard deviation of 32.3%. Our preliminary data indicate that the compound is only weakly taken up following oral administration.

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