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# EVALUATION OF IMMUNOSORBENTS FOR THE ANALYSIS OF SMALL MOLECULES

## ISOLATION AND PURIFICATION OF CYTOKININS

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SUMMARY

This paper describes the preparation and evaluation of immunosorbents for the isolation of cytokinins. The nature of both the solid support and the linkage chemistry affected the non-specific adsorption of sample contaminants and the characteristics of the immobilized antibody. All of the immunosorbents investigated provided sufficient purification of cytokinins for high-performance liquid chromatographic analysis with no further clean-up. This demonstrates a rapid and powerful purification method for small molecules for which antibodies can be generated. As a consequence, the procedures described for the immobilization of cytokinin antibodies are generally applicable to the preparation of immunosorbents.

## INTRODUCTION

In recent years procedures for trace organic analysis have been improved significantly by employing techniques such as high-performance liquid chromatography (HPLC) or capillary gas chromatography. The resolution that can be obtained with these separation modes has made it possible to separate compounds with only minor differences. These methods and most pre-column protocols are non-specific in that they rely on parameters such as charge or hydrophobicity for separation. Many complex samples contain large numbers of background contaminants which occur at high concentrations and cannot be separated from the trace molecules of interest with these relatively non-specific techniques. Consequently, separation methods which are effective in analysis of standards are rarely useful in analysis of trace molecules in real samples.

Affinity techniques offer an attractive solution to this problem. By employing a biospecific ligand on a suitable column support, it is possible to capture a single compound or a family of compounds from a complex mixture. With the appropriate eluent, the molecule of interest can then be released in a highly purified fraction suitable for analysis by physico-chemical or immunological approaches.

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Traditionally, separations based on affinity interactions have been used for the isolation and purification of a variety of large molecules[1,2]. Classical affinity separations have been limited to those molecules with naturally occurring intermolecular interactions. Examples include lectins, nucleic acids, coenzymes, and various dyes. As a result, affinity separations are constrained in practice by the inherent characteristics of these interactions. The special case of immunoaffinity binding is more generally useful for affinity based separations since antibodies can be raised against many molecules for which other affinity interactions have not been described. In addition, reagent antibodies can be generated with a range of specificity and affinity which allow some selection of the character of the interaction. As with most affinity techniques, immunoaffinity has had the greatest impact on the isolation and purification of macromolecules. The parameters governing the interactions of large molecules in preparative affinity chromatography have been described[3].

There are a limited number of examples of similar separation techniques for small molecules[4–10]. This is surprising, as reagent antibodies have been raised against many small molecules (usually as haptens on larger carrier molecules such as bovine serum albumin) and used in immunological assays. A powerful application of many of these same antibodies would be immunoaffinity separations for the purification of small molecules.

In this paper we describe the development of immunosorbents that are used to isolate endogenous plant growth regulators (cytokinins) from plant tissue extracts. The general application of immunosorbents for group selective purification of small molecules is discussed.

## EXPERIMENTAL

### *Immunogen preparation*

The plant hormones zeatin riboside [6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-9-$\beta$-D-ribofuranosylpurine] and dihydrozeatin riboside [6-(4-hydroxy-3-methylbutylamino)-9-$\beta$-D-ribofuranosylpurine] are too small to be immunogenic and must be conjugated to a protein large enough to elicit an immune response. Consequently, polyclonal antisera were raised in rabbits using conjugates of zeatin riboside (ZR) or dihydrozeation riboside (DHZR) and bovine serum albumin (BSA). The conjugates were prepared by periodate oxidation of the ribose ring followed by coupling of the resulting diketone to the epsilon amino group of BSA lysine residues[11]. The aldimine adduct linkage was reduced with sodium borohydride to stabilize the conjugate. The molar coupling ratio of ZR or DHZR to BSA was determined to be 7:1 by measurement of the change in absorbance of the BSA at 280 nm after conjugation[11].

### *Immunization protocol*

Antigens were initially introduced into rabbits in Freund's complete adjuvant by subcutaneous and intramuscular injection with 1 mg of conjugate (multiple sites). Rabbits were boosted every three weeks with 1 mg of conjugate in Freund's incomplete adjuvant. Then 10–14 days following the second boost, the serum was checked for antibody titre. Rabbits displaying a measurable titre were boosted with 200 $\mu$g of conjugate approximately two weeks later. Boosts were given on a three-to-four

week basis and the titre was checked *ca.* 10 days after each boost. In many rabbits a serum titre of 1:12 000 was observed with a few showing titres of 1:60 000. The rabbit serum against the individual hormones was pooled and then retitred. The pooled serum used in this study for column preparation and evaluation had a titre of 1:51 200 for anti-ZR and 1:26 500 for anti-DHZR.

*IgG purification*

The immunoglobulin G (IgG) fraction was isolated by passing the serum over a Protein A Sepharose column (Sigma, St. Louis, MO, U.S.A.). The column was washed with several column volumes of phosphate buffered saline (0.01 *M* phosphate–0.15 *M* sodium chloride, pH 7.4) and then the IgG eluted with 0.58% acetic acid–0.15 *M* sodium chloride. The IgG fraction was then dialyzed against phosphate buffered saline (PBS) and concentrated prior to immobilization on the various supports. The IgG concentration was determined spectrophotometrically at 280 nm.

*Soluble antibody characterization*

After isolation of the IgG fraction, the equilibrium binding constant ($K_a$) was

TABLE I

CROSS-REACTIVITY OF TWO POLYCLONAL ANTISERA TO CYTOKININS AND ADENINE ANALOGS

Cross-reactivities were measured by a competitive radioimmunoassay[11] in which the concentration of cross-reactant which inhibited the binding of the primary antigen by 50% was determined.

| *Cross-reactant* | *Percent cross-reactivity* | |
|---|---|---|
| | *Anti-ZR** | *Anti-DHZR*** |
| 2-Isopentenyl adenosine | 0.48 | 2.70 |
| 2-Isopentenyl adenine | 0.09 | 0.52 |
| Zeatin riboside | 100.00 | 0.42 |
| Zeatin | 35.70 | 0.06 |
| Dihydrozcatin riboside | 0.53 | 100.00 |
| Dihydrozeatin | 3.04 | 24.00 |
| Isopentenyl adenosine monophosphate | 0.06 | 0.32 |
| Zeatin riboside monophosphate | 26.50 | 0.15 |
| Kinetin | 0.00 | 0.46 |
| Zeatin-9-glucoside | 21.95 | 0.34 |
| 2-Methylthiol isopentenyl adenosine | 0.02 | 0.61 |
| 2-Methylthiol zeatin | 1.34 | 0.09 |
| Benzylaminopurine | 0.04 | 0.32 |
| Benzylaminopurine riboside | 0.14 | 4.84 |
| Pentylpurine riboside | 0.04 | 18.80 |
| Isoamylpurine riboside | 0.03 | 32.00 |
| Adenosine triphosphate | 0.00 | 0.00 |
| Adenine | 0.00 | 0.00 |
| Adenosine | 0.00 | 0.00 |

* Anti-ZR, polyclonal antiserum raised against zeatin riboside-BSA conjugate.

** Anti-DHZR, polyclonal antiserum raised against dihydrozeatin riboside-BSA conjugate.

determined by competitive radioimmunoassay (RIA)[12]. Cross-reactivity of the polyclonal antibodies to a number of cytokinins and cytokinin analogs was also determined by competitive RIA (Table I). The cross-reactivity was determined by measuring the molar concentration of competing cytokinin necessary to reduce the binding of [$^3$H]-antigen by 50%.

*Antibody immobilization*

*CNBr-activated Sepharose 4B.* A 1-g sample of freeze dried gel (Pharmacia, Piscataway, NJ, U.S.A.) was reswelled in 1 m*M* hydrochloric acid and transferred to a sintered glass funnel. The gel was filtered and washed with 1 m*M* hydrochloric acid. Immediately prior to use, the gel was washed with coupling buffer (0.1 *M* sodium bicarbonate–0.5 *M* sodium chloride, pH 8.3), drained and rapidly transferred to 4 ml of coupling buffer which contained the IgG fraction. Typical amounts of IgG used were 5 mg/ml of gel. The solution was mixed end-over-end in a 15 ml polypropylene test-tube for 2 h at room temperature. The gel was then spun down at low rpm (300 *g*) and the buffer removed and saved for the determination of coupling efficiency. After transfer to a buffer containing a blocking agent (1 *M* ethanolamine, pH 8.0) the gel was mixed end-over-end overnight at 4°C, then washed with coupling buffer followed by acetate buffer (0.1 *M* acetate–0.5 *M* sodium chloride, pH 4.0). This was followed by a final wash with coupling buffer. Finally, the gel was poured into a column and washed with PBS. Typical coupling efficiencies for this gel were 85–90%.

*Affi-Gel 10.* Affi-Gel 10 (Bio Rad, Richmond, CA, U.S.A.) was transferred as a slurry to a sintered glass funnel and drained. Then the gel was washed with isopropyl alcohol followed by several washes of cold water. In a 15-ml polypropylene test-tube the gel was mixed with 4.0 ml of coupling buffer (0.1 *M* sodium bicarbonate, pH 8.0) containing the IgG. Typical amounts of IgG that were used in this procedure were 35 mg/ml of gel. After mixing end-over-end for 1 h at room temperature, the gel was centrifuged at low rpm (300 *g*) and the supernatant collected for the determination of coupling efficiency. Blocking buffer was then added to the tube (1 *M* ethanolamine, pH 8.0) and mixed gently for one hour at room temperature. The gel was transferred to a column and washed with coupling buffer until the OD (280 nm) reading returned to baseline. The column was then equilibrated in PBS and stored at 4°C until use. Coupling efficiencies of greater than 90% were routinely obtained.

*Glycophase silica.* Following the protocol of Roy *et al.*[13], 1 g of silica (Nugel P, GP-500, 500 A porosity 200–400 mesh, Diagnostic Specialties, Metuchen, NJ, U.S.A.) was added to 2 ml of 1% sodium metaperiodate and agitated for 30 min at room temperature. The activated silica was then washed in a sintered glass funnel with cold distilled water and transferred to a 15-ml polypropylene test-tube that contained coupling buffer (0.1 *M* phosphate–0.1 *M* sodium chloride, pH 7.0) and the purified IgG (25 mg). To this mixture 1 mg of cyanoborohydride was added and the solution mixed end-over-end for 24 h at 4°C. The silica was then washed thoroughly with cold coupling buffer and the washes were collected for protein determinations. Uncoupled sites were blocked by suspending the silica in 4 ml of 1 *M* ethanolamine (pH 8.5) with 1 mg of cyanoborohydride. The suspension was mixed end-over-end overnight at 4°C. The silica was then washed with cold coupling buffer followed by a wash with PBS, poured into a column and stored at 4°C. Typical coupling efficiency of 80–85% was obtained.

*Fast-Flow Sepharose CL-4B periodate coupling.* Following the protocol of Roy *et al.*[13], 4.0 ml of Fast-Flow Sepharose CL-4B (Pharmacia, Piscataway, NJ, U.S.A.) were added to 2.0 ml of 1% sodium metaperiodate and agitated for 30 min at room temperature. The activated gel was then washed on a sintered glass funnel with cold water, and added to a 15 ml polypropylene test-tube that contained coupling buffer (0.1 *M* phosphate–0.1 *M* sodium chloride, pH 7.0), 25 mg of IgG, and 1 mg of cyanoborohydride. The solution was mixed end-over-end for 24 h at 4°C and then washed with cold coupling buffer over a sintered glass funnel. The filtrate was collected for the determination of coupling efficiency. Uncoupled activated sites were blocked by suspending the gel in 5 ml of 1 *M* ethanolamine (pH 8.0) and adding 1 mg of cyanoborohydride. This mixture was tumbled for 24 h at 4°C and then washed with PBS. The modified gel was then poured into a column and stored in PBS at 4°C until use. Coupling efficiencies of 70% have been obtained with this method.

*Carbonyl diimidazole (CDI) coupling of Fast-Flow Sepharose CL-4B.* Following the protocol of Bethel *et al.*[14], 4.0 ml of the gel were washed over a sintered glass funnel with 10 ml of water, then 10 ml of 50% water–acetone, followed by four 5-ml washes of 100% acetone. The acetone moist cake was then transferred to a 15-ml polypropylene test-tube which contained 5 ml of 0.3 *M* CDI. The mixture was agitated for 15 min at room temperature. The suspension was then filtered and washed with acetone followed by coupling buffer (0.1 *M* phosphate–0.1 *M* sodium chloride, pH 7.0). The buffer washed cake was transferred to another test-tube which contained 8.0 ml of coupling buffer and 53 mg of IgG. The suspension was agitated for 24 h at 4°C. The following day the suspension was centrifuged and the supernatant collected for the determination of coupling efficiency. Active sites were blocked by suspending the gel in 1 *M* ethanolamine (pH 8.5) and agitating overnight. The following day the gel was washed with PBS and poured into a column. The coupling efficiencies with this approach have been greater than 90%.

*Ultraffinity-EP.* Following the product guidelines (Beckman, Berkeley, CA, U.S.A.), 25 mg of protein A purified antibody were dissolved in 30 ml of 1.0 *M* phosphate buffer (pH 7.0) and recycled through the Ultraffinity-EP HPLC column (15 cm × 0.46 cm I.D., 10-$\mu$m particle diameter) for 12 h. The column was washed with PBS prior to use. Coupling efficiency was greater than 90%.

*Immunoaffinity control column.* In order to examine non-specific binding of cytokinins to the immobilized IgG protein or the column support material, a control column was prepared with non-specific rabbit IgG (Sigma) and CNBr-activated Sepharose 4B. The coupling protocol was identical to that described earlier for CNBr-activated Sepharose 4B.

*Column pretreatment*

All the columns were pretreated with 100% methanol before use, to eliminate the effects of any methanol sensitive sub-population of antibodies on capacity studies. When the columns were not used for long periods of time they were stored in PBS–0.01% azide. The columns used for this study were constructed of polypropylene (Isolab, Akron, OH, U.S.A.).

*Immobilized antibody evaluation*

The dynamic capacity of each column was estimated at a fixed flow-rate by

applying increasing amounts of the appropriate cytokinin, which contained a known amount of tritiated internal standard ([$^3$H]-ZR dialcohol, 43Ci/mmole; [$^3$H]-DHZR, 58Ci/mmole; Amersham, Arlington Hts, IL, U.S.A.) and determining the amount of radiolabel retained by the column. A plot of radiolabel retained by the column *vs.* amount of cytokinin applied to the column was examined graphically, and the dynamic capacity determined by extrapolating back to the point at which the recovery dropped from a constant value.

Once the dynamic column capacity was determined, the $K_a$ of the immobilized antibody was measured by a modification of the RIA used to estimate the $K_a$ of the antibody in solution[12]. A measured amount of gel, corresponding to a defined capacity was suspended in PBS–0.5 *M* mannitol. The mannitol was included in the buffer to retard sedimentation of the immunosorbent by increasing the viscosity and density of the suspension buffer. Suspended immunosorbents were then used in the RIA (Fig. 1) in place of the soluble antibody. This method allowed estimation of the $K_a$ values of many of the immunosorbents. The high density of the silica-based supports resulted in rapid settling of the immunosorbent and in poor precision of the assay. Cross-reactivity of the anti-ZR immunosorbents to DHZR (Table II) was determined as described earlier for the soluble antibody.

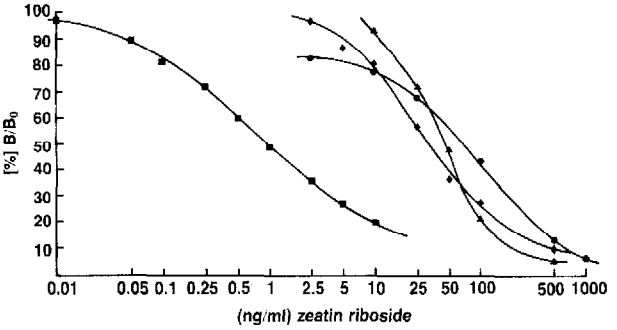


Fig. 1. Competetive radioimmunoassay of immobilized anti-zeatin riboside (ZR) antisera. Binding of [$^3$H]ZR dialcohol to antisera linked to Affi-Gel 10 (■), CNBr-activated Sepharose 4B (◆), carbonyl diimidazole-linked Fast-Flow Sepharose CL-4B (●) or periodate-linked Fast-Flow Sepharose CL-4B (▲) in the presence of various amounts of ZR is shown as the percentage of radiolabeled ZR dialcohol bound to the immunosorbents in the absence of ZR ($B/B_0$).

*Extraction of leaf tissue*

In order to evaluate the immunosorbent columns for purification of cytokinins from plant samples, a large plant sample was prepared for comparative use with all columns. A 5-g sample of soybean trifoliolate leaves was harvested from greenhouse grown plants and homogenized in 75 ml of cold (−20°C) 80% methanol containing 10 mg/l butylated hydroxytoluene and 500 mg/ml citrate[15]. The homogenate was filtered through miracloth and centrifuged at 15 000 *g* for 15 min. The resulting supernatant was reduced to the aqueous phase and brought to 50 ml with PBS. In all cases, 2.0 ml of the stock leaf extract (equivalent to 200 mg of leaf tissue) were applied to the immunosorbent column. Just prior to application, the sample was spiked with both unlabelled and labelled internal standards (ZR, 31 ng and DHZR, 24 ng plus 44 000 dpm [$^3$H]-DHZR) and filtered through a prewashed 0.2-$\mu$m filter.

TABLE II

COMPARISON OF BINDING CONSTANTS ($K_a$) AND CROSS-REACTIVITIES TO DHZR* OF IMMOBILIZED AND SOLUTE ANTIBODIES RAISED AGAINST ZEATIN RIBOSIDE-BSA CONJUGATES

Binding constants and percent cross-reactivity were determined by radioimmunoassay[11]

| *Immunosorbent* | *Binding constant for ZR*** $K_a$ ($M^{-1}$) | *Percent cross-reactivity to DHZR* |
|---|---|---|
| Fast-Flow Sepharose CL-4B (carbonyldiimidazole method) | $5.3 \cdot 10^{10}$ | 1.65 |
| Fast-Flow Sepharose CL-4B (periodate method) | $9 \cdot 10^{10}$ | 1.54 |
| CNBr-activated Sepharose 4B | $1.2 \cdot 10^{11}$ | 2.5 |
| Affi-Gel 10 | $4.6 \cdot 10^{12}$ | 1.56 |
| Soluble antibody | $1.4 \cdot 10^{13}$ | 0.53 |

* DHZR, dihydrozeatin riboside.
** ZR, zeatin riboside.

*Evaluation of columns for plant sample purification*

Columns were evaluated for recovery of cytokinin internal standards and for non-specific adsorption of molecules in the plant sample. Different immunosorbent supports were compared under conditions of equivalent maximum capacity (2μg ZR) and also under conditions of equal bed volume (0.5 ml). Under conditions of constant column capacity the samples were applied to the columns in one of two ways. The 2.0-ml sample was applied directly or it was diluted 10-fold prior to application. The sample was diluted in order to evaluate the effects of the sample matrix on cytokinin recovery and on non-specific adsorption.

In all cases samples were drawn through the columns at 1.5 ml/min with a peristaltic pump, then washed with 15.0 ml of PBS, followed by 15.0 ml of deionized, distilled water. Material retained by the immunosorbent columns after washing, was eluted with 15.0 ml of methanol by gravity flow. The methanol eluate was dried under reduced pressure, then dissolved in deionized distilled water prior to analysis by HPLC.

*Column flow-rate studies*

A study was conducted to determine to what extent flow-rate or, more importantly, linear velocity affected capture efficiency. Immunosorbent columns of varying capacities and bed volumes were selected. To each column a constant amount of analyte in PBS (spiked with the appropriate radiolabeled internal standard) was applied and the flow-rate through the column varied. PBS was used as the mobile phase and 8–10 column volumes were collected before the methanol elution. Both the wash and the methanol eluate were collected for counting. The recovery was determined by scintillation counting of the two fractions. The amount of cytokinin added to each column was approximately one-half the capacity of the column to avoid overloading the columns.

*Extraction of soybean seeds*

Whole soybean seeds, 3.0 mm in length, were pulverized in a 1.5-ml microcentrifuge tube with a glass pestle and extracted in 1.0 ml of the same methanolic buffer used for leaf extraction. After centrifugation (13 000 *g*) for 5 min, the extract was resuspended in extraction buffer, centrifuged, and the supernatants combined. This sample was then purified as described for leaf extracts.

*HPLC separation of cytokinins*

Samples purified on immunosorbent columns or standards were separated by reversed-phase HPLC on Ultrasphere ODS (Altex, 5 μm, 25 cm × 0.46 cm I.D.) at 45°C. Cytokinins were eluted isocratically with 11% acetonitrile (v/v) in 0.01 *M* acetic acid–0.005 *M* triethylamine (pH 3.35) at 1.3 ml/min and detected by absorbance at 272 nm. At the elution time of DHZR, the column eluate was collected, and recovery of [$^3$H]-DHZR determined by liquid scintillation counting.

## RESULTS AND DISCUSSION

*Conjugation efficiency and column capacity*

All of the linkage methods resulted in high efficiency conjugation (75–95%) of the proteins to the supports. Antibodies were conjugated to the supports over a range of concentrations (Table III). Recommended protein densities from the manufacturer's literature were used where available, or in some cases, IgG was immobilized at higher densities. Comparison of column capacities (μg ZR per ml support) and protein loadings (mg IgG per ml support) on the five supports (Table III) shows that there was not a common capacity per unit protein (specific capacity). The Sepharose supports all had similar specific capacities of *ca.* 0.3 μg of ZR per mg of immobilized

TABLE III

DYNAMIC COLUMN CAPACITY AND PROTEIN LOADING OF ANTI-ZR* IMMUNOSORBENTS

Dynamic column capacities were determined by measuring the retention by columns of [$^3$H]ZR dialcohol internal standard in the presence of unlabeled ZR. Protein loading was estimated by measuring the amount of protein remaining in solution by determining the absorbance at 280 nm after conjugation reactions.

| *Immunosorbent* | *Protein loading (mg/ml)* | *Dynamic column capacity (μg ZR/ml)* | *Specific capacity (μg ZR/mg protein)* |
|---|---|---|---|
| Fast-Flow Sepharose CL–4B–CDI** | 13.7 | 4.0 | 0.29 |
| Fast-Flow Sepharose CL–4B–periodate | 4.0 | 1.2 | 0.30 |
| CNBr Sepharose 4B | 4.2 | 1.3 | 0.31 |
| Glycophase Silica | 13.9 | 2.4 | 0.17 |
| Affi-Gel 10 | 28.0 | 4.0 | 0.14 |

* ZR, zeatin riboside.

** CDI, carbonyldiimidazole linkage.

IgG. This value was similar for the Sepharose supports regardless of the protein density or the linkage chemistry. Affi-Gel 10 and glycophase silica had specific capacities approximately half that of the sepharose gels. Since only a single protein density was tested for most of the linkage–support combinations, these results do not necessarily represent an inherent characteristic of the supports.

Nevertheless the data do indicate the potential for optimizing the specific column capacity. This is an important consideration especially in situations where the antibody supply is limited. In such cases the most efficient use of the antibody would be the construction of high specific capacity columns.

The linkage methods investigated in this work rely on attachment to the support through random linkage with the epsilon amino groups of the protein lysyl residues. Other derivatization strategies could be employed that would direct the antigen binding sites away from the support matrix. Specific reactions coupling the Fc portion of the antibody to the solid support is one approach to achieving a more specific protein-support linkage[16,17]. Other approachs are currently being investigated in our lab as well.

Since polyclonal sera were employed for construction of the immunosorbents listed in Table III, only a portion of the IgG immobilized on the supports was directed against cytokinins. When antibody titres are high (as is the case here), the entire IgG fraction can be immobilized. If antibody titres are low, however, an additional affinity isolation on an antigen column could be valuable. This would allow construction of columns with high specific capacity and help reduce the gel volume necessary to have an effective immunosorbent. Low titre antisera that was not antigen-selected in this manner would require immobilizing more protein and increasing gel volume to produce columns of adequate capacity. A monoclonal antibody with a high $K_a$ that could withstand the rigors of the elution scheme would eliminate the need for separation of antigen-specific antibodies from polyclonal sera. A population of uniform and specific antibodies would allow the development of high specific capacity columns.

As a consequence of the different specific capacities of the antibody on different supports and of the different protein–gel conjugation ratios, the immunosorbents exhibited different column capacities. The capacities ranged from 1.2 to 4.0 $\mu$g of ZR per ml of immunosorbent (Table III). In order to evaluate the capture efficiency of each of the immunosorbents it was necessary to compare the columns on the basis of identical column capacity (described later). The non-specific adsorption characteristics of the columns were evaluated with columns of identical bed volume.

The capacity of the immunoaffinity columns remained stable in spite of the nature of the samples and of the elution solvent. Methanol was chosen as the elution solvent since it removed standards of both ZR and DHZR from the columns in smaller volumes than a variety of chaotropic and ionic agents. After the first time methanol was passed over the immunosorbents, no reduction in capacity was observed. The immobilized antibodies were unaffected by repeated treatment with methanol and could be stored indefinitely in PBS–0.01% azide (w/v) at 4°C.

*Effect of coupling on antibody performance*

Immobilization of the anti-ZR IgG resulted in a reduction of the $K_a$ from the value for the soluble antibody (Fig. 1, Table II). Antibody immobilized on Affi-Gel

10 only displayed a three-fold reduction in $K_a$, while the antibody immobilized on Sepharose supports had $K_a$ values reduced by more than 100. It was not possible to determine the $K_a$ of the antibody immobilized to silica due to the physical problems in handling the support. Of the four sorbents evaluated for $K_a$, only the Affi-Gel 10 linkage included a spacer molecule between the antibody and the solid support. The presence of this spacer between the antibody and the support affords more conformational freedom to the antibody and is more likely responsible for the higher $K_a$ value of the Affi-Gel sorbent than any differences between the Affi-Gel and Sepharose supports.

In addition to the reduction in affinity imparted by immobilization, the cross-reactivity of the antibody to another cytokinin (DHZR) was increased at least three-fold (Table III). Relaxation of the antibody specificity was not an unexpected result since the conformation of the antibodies, and therefore the configuration of the antigen binding sites, could be expected to change due to covalent linkage to the rigid support. The cross-reactivity of the sorbent can be advantageous if isolation of a group of closely related molecules is the primary objective of the separation. This is particularly true when suitable separation methods can be applied to resolve the individual molecules of interest after the immunosorbent isolation.

The molar capacities of the anti-ZR immunosorbents for DHZR were nearly identical to the capacities for ZR. In addition, recovery of other molecules with significant cross-reactivities was relatively high for both the DHZR and ZR immunosorbents (data not shown). This suggests that the observed cross-reactivity is an inherent characteristic of the whole immobilized antibody population, rather than an indication of a small sub-population of antibodies which recognizes a cross-reactant. This phenomenon has been observed in populations of antibodies raised against other structurally related haptens[18]. While the capacity of the column may be experimentally determined to be similar for a number of cross-reacting species, the recovery of a cross-reactant from a sample can be lower than that of the primary antigen. This is due to the lower $K_a$ value of the antibody for the cross-reacting species than for the primary antigen. Reduced recoveries may occur if a solute is present at sufficiently low concentrations, or if column capacity is low and cross-reactants are unable to compete for limited binding sites with molecules for which the antibodies have higher $K_a$ values. Therefore it is advisable to prepare columns with binding capacities well in excess of the amount of solute expected in a single sample.

*Non-specific binding*

Results obtained from our initial investigations of immunosorbents for cytokinins[9,19] indicated that the non-specific adsorption of molecules from a crude plant sample was responsible for most of the background in the subsequent analyses. Non-specific interactions of molecules in samples might be expected in cases where residual reactive groups from the immobilization reaction are still present in the gel and can covalently interact with the sample. They may also arise in cases where polar or charged moieties are present in the linkage group, resulting in secondary reactions. These possibilities exist in the case of CNBr-activated polyhydroxy supports. In addition to the reactive cyanate ester which is the primary reactive group for immobilization of the protein, less reactive imidocarbonates are formed. Hydrolysis of the cyanate ester results in formation of carbamates which can undergo polar or ionic

interactions with a sample depending on the pH in the column[20]. The isourea linkage formed between the cyanate ester and primary amino groups is also positively charged at neutral pH leading to another possible secondary interaction.

In order to reduce the non-specific interaction of the plant sample with the immunosorbent, activated immunosorbents were reacted with a blocking agent (*e.g.* ethanolamine) to neutralize residual reactive groups on the gels. Linkage chemistries other than isourea conjugates from CNBr-activated supports were also evaluated with respect to residual reactivity and polar interactions. These linkages included carbonyldiimidazole, N-hydroxysuccinimide, and periodate oxidation. A number of supports were evaluated with these linkage chemistries to determine the inherent interactions of the support with contaminants in the samples, and the ability to capture ZR and DHZR from a sample matrix.

The contribution of a non-cytokinin specific population of IgG to adsorption and retention of cytokinins was determined with non-specific rabbit IgG immobilized to CNBr Sepharose. Standards of ZR and DHZR were applied to this IgG sorbent and eluted in the same manner as for anti-ZR columns (data not shown). No retention of ZR or DHZR by this column was detected. Retention of the cytokinins by the immunosorbents was due solely to the presence of the immunospecific IgG fraction generated in response to the immunogen (ZR-BSA).

*Column performance*

The non-specific interactions of molecules in the plant samples, column capacity and recovery characteristics were evaluated for the anti-ZR immunosorbents. Each of the supports and linkage chemistries had inherent characteristics for solute capacity and non-specific interactions with solute molecules in the sample. These interactions were evaluated by observing the impurities in the HPLC chromatograms of ZR and DHZR after immunoaffinity separation (Figs. 2–4). Nearly all of the impurities were polar in nature and were well separated from the cytokinins. One persistent impurity, present in both samples and standards had a capacity factor ($k'$) slightly higher than that of DHZR. This impurity was not specific to the immunosorbents and appeared to be present in the chromatographic system. No other impurities occurred with $k'$ values similar to the cytokinins ZR or DHZR in any of the immunosorbent separations.

Inspection of the chromatograms of the purified 2.0-ml leaf samples (Fig. 2) illustrates the differences in non-specific adsorption characteristic of the five immunosorbents. The Fast-Flow Sepharose supports provided the best purification of 2.0-ml samples since neither retained significant amounts of polar material which might interfere with subsequent detection of the cytokinins. This might be expected as the periodate and CDI coupling procedures essentially link the protein directly to the support. This should eliminate background adsorption associated with linker molecules.

While no single component interfered with detection of the cytokinins after purification on the other three supports, they retained considerably higher amounts of polar material. This is not a surprising result since the N-hydroxysuccinimide (Affi-gel) and CNBr (Sepharose 4B) chemistries result in polar linkages or have the potential for side reactions which result in polar or charged groups. The Glycophase silica has a high concentration of unreacted silanols available for polar interactions with solutes in the sample matrix.

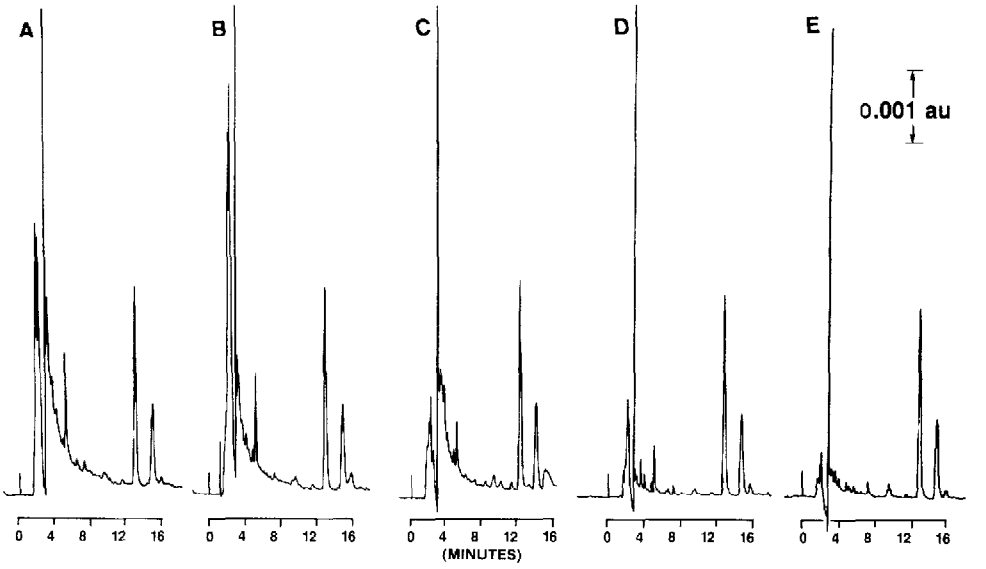


Fig. 2. Separation of zeatin riboside (ZR) and dihydrozeatin riboside (DHZR) from immunopurified soybean leaf extracts by HPLC. A 2-ml sample (200 mg equivalent leaf fresh weight) of leaf extract was purified with anti-ZR immunosorbent columns (2 $\mu$g ZR capacity) of Affi-Gel 10 (A), CNBr-activated Sepharose 4B (B), Glycophase Silica (C), CDI-linked Fast-Flow Sepharose CL-4B (D), or periodate-linked Fast-Flow Sepharose CL4B (E). Immunopurified samples were separated on Ultrasphere ODS (5 $\mu$m, 25 cm $\times$ 0.46 cm I.D.) with 0.01 *M* acetic acid–0.005 *M* triethylamine (pH 3.35) at 1.3 ml/min (45°C) and detected by absorbance at 272 nm.

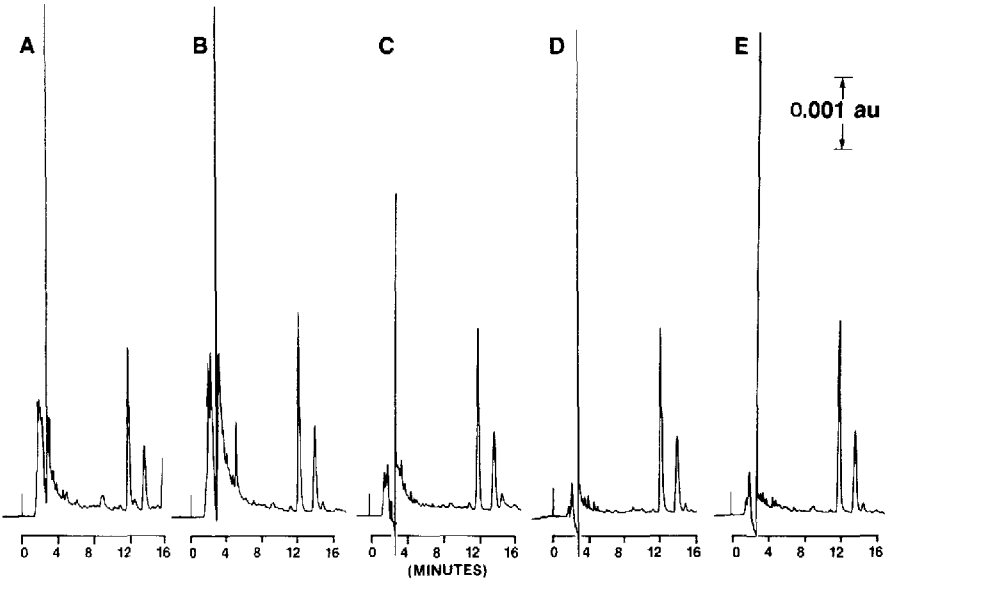


Fig. 3. Separation of zeatin riboside (ZR) and dihydrozeatin riboside (DHZR) from immunopurified soybean leaf extracts by HPLC. A 2-ml sample (200 mg equivalent leaf fresh weight) of leaf extract was diluted to 20 ml and purified with anti-ZR immunosorbent columns (2 $\mu$g ZR capacity) of Affi-Gel 10 (A), CNBr-activated Sepharose 4B (B), Glycophase Silica (C), CDI-linked Fast-Flow Sepharose CL-4B (D), or periodate-linked Fast-Flow Sepharose CL-4B (E). Immunopurified samples were separated on Ultrasphere ODS (5 $\mu$m, 25 cm $\times$ 0.46 cm I.D.) with 0.01 *M* acetic acid–0.005 *M* triethylamine (pH 3.35) at 1.3 ml/min (45°C) and detected by absorbance at 272 nm.

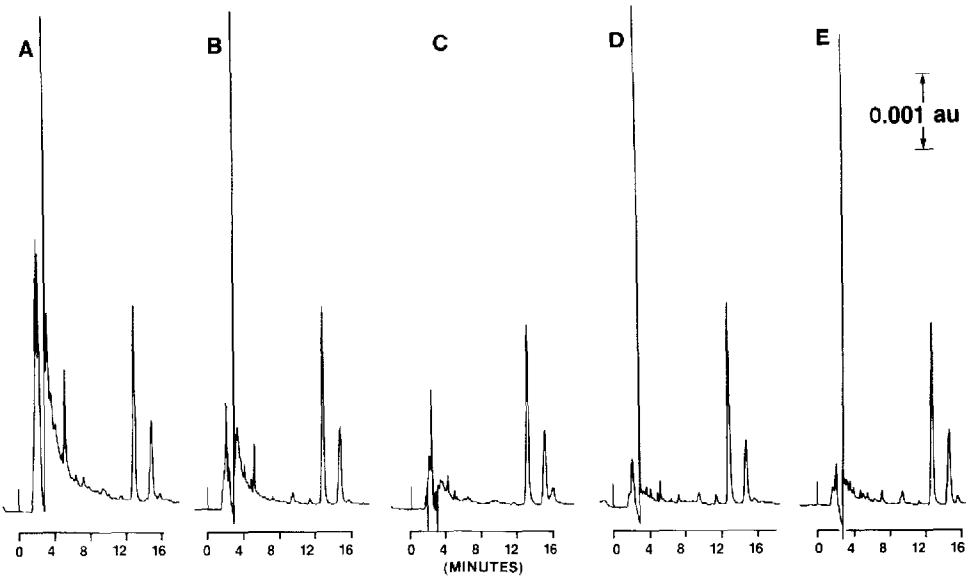


Fig. 4. Separation of zeatin riboside (ZR) and dihydrozeatin riboside (DHZR) from immunopurified soybean leaf extracts by HPLC. A 2-ml sample (200 mg equivalent leaf fresh weight) of leaf extract was purified with anti-ZR immunosorbent columns of 0.5 ml bed volume, Affi-Gel 10 (A), CNBr-activated Sepharose 4B (B), Glycophase Silica (C), CDI-linked Fast-Flow Sepharose CL-4B (D), and periodate-linked Fast-Flow Sepharose CL-4B (E). Immunopurified samples were separated on Ultrasphere ODS (5 $\mu$m, 25 cm $\times$ 0.46 cm I.D.) with 0.01 *M* acetic acid–0.005 *M* triethylamine (pH 3.35) at 1.3 ml/min (45°C) and detected by absorbance at 272 nm.

Comparison of the chromatograms of the 2.0-ml samples (Fig. 2) with those of identical plant samples diluted to 20.0 ml (Fig. 3) shows the effect of concentration of the sample on non-specific adsorption. The 10-fold reduction in sample concentration clearly reduced non-specific adsorption on the Glycophase silica and Affi-Gel 10 supports. Neither of the Fast-Flow Sepharose based supports showed much reduction in non-specific binding with the dilute sample. Given the already low background in the more concentrated 2.0-ml samples, it would have been difficult to detect a reduction in non-specific adsorption at the sensitivity employed. The CNBr-activated Sepharose was the least sensitive to sample dilution and showed the least favorable adsorption characteristics.

Reducing the sample concentration also resulted in a reduction in recovery of the DHZR (Table IV). Sample recovery on Affi-Gel 10, and periodate linked Fast-Flow Sepharose were the most sensitive to sample dilution, while the CDI-linked Fast-Flow Sepharose was essentially unaffected. Although the reduction in recovery is a point to consider, the recoveries observed were quantitative despite sample dilution. In the case of viscous samples it may be advisable to dilute the sample to minimize background adsorption on some of the supports investigated here.

The chromatograms of plant samples which were immunoaffinity purified on columns of identical bed volume (Fig. 4) confirmed that both the chemistry of the linkage and the bulk of available immunosorbent contribute to the amount of non-specific adsorption. Affi-Gel-10 and CNBr-activated Sepharose displayed the most contribution to sample background on a volume basis. The Fast-Flow Sepharose

sorbents had the lowest inherent non-specific adsorption and the Glycophase Silica was intermediate. Reduction of column volume to 0.5 ml resulted in lower column capacities for the CNBr-activated Sepharose, Glycophase Silica and Fast-Flow Sepharose sorbents than for the previous comparison. These reduced capacities were manifested in lowered recoveries of [$^3$H]-DHZR for all three columns (Table IV). Since these columns displayed cross-reactivities for DHZR of *ca.* 2% of ZR, the recovery of [$^3$H]-DHZR was a conservative estimate of the capture efficiency of ZR.

Both ZR and DHZR were minor components of the leaf extract used to evaluate the immunosorbents. These molecules are labile in plant extracts and for this reason small amounts of standards were added to the samples just prior to purification to provide uniform samples for post-purification comparison of the immunosorbents. Typically, isolation and determination of cytokinins from plant samples requires up to three consecutive HPLC separations[21]. By comparison, all of the anti-ZR immunosorbents provided sufficient sample purification to permit detection of both ZR and DHZR in a single isocratic reversed-phase separation (Figs. 2–4). The highly selective nature of the immunosorbents is evident by comparing the chro-


Fig. 5. Separation of soybean leaf extract (200 mg fresh weight equivalent) by HPLC without immunosorbent purification. Sample was separated on Ultrasphere ODS (5 μm, 25 cm × 0.46 cm I.D.) with 0.01 *M* acetic acid–0.005 *M* triethylamine (pH 3.35) at 1.3 ml/min (45°C) and detected by absorbance at 272 nm. Full scale absorbance is 10-fold higher than for Figs. 2–4.

TABLE IV

EFFECT OF SAMPLE CONCENTRATION AND COLUMN BED VOLUME ON RECOVERY OF [$^3$H]DHZR* BY ANTI-ZR** IMMUNOSORBENT COLUMNS

The percentage of radiolabeled [$^3$H]DHZR which was retained by the columns and subsequently eluted with methanol was determined by scintillation counting. Samples were applied at 1.5 ml/min and eluted by gravity flow.

| *Immunosorbent* | *Column bed volume (ml)* | *Recovery sample volume (%)* | | *Column bed volume (ml)* | *Recovery sample volume (%) 2.0 ml* |
|---|---|---|---|---|---|
| | | *2.0 ml* | *20.0 ml* | | |
| Fast-Flow Sepharose CL-4B–CDI*** | 0.5 | 91 | 89 | 0.5 | 91 |
| Fast-Flow Sepharose CL-4B–periodate | 1.7 | 100 | 82 | 0.5 | 77 |
| CNBr Sepharose 4B | 1.5 | 100 | 91 | 0.5 | 89 |
| Glycophase Silica | 0.8 | 97 | 86 | 0.5 | 91 |
| Affi-Gel 10 | 0.5 | 90 | 74 | 0.5 | 90 |

* [$^3$H]DHZR, [$^3$H] dihydrozeatin riboside.
** ZR, zeatin riboside.
*** CDI, carbonyldiimidazole linkage.

matograms of an unpurified plant sample (Fig. 5) with the chromatograms of the immunosorbent purified samples (Figs. 2–4). No major contaminants from the crude plant extract (Fig. 5) interfered with the detection of ZR or DHZR after a single immunosorbent purification. Even three chromatographic separations do not provide this level of sample purification.

*Flow-rate studies*

The first immunosorbent columns prepared in our laboratory were CNBr-coupled Sepharose. Given the back-pressure limitations with this type of resin, flow-rates through the 0.9-cm diameter columns were restricted to 1.5 ml/min and below. At these flow-rates nearly 100% capture efficiency was observed. As alternate supports which could tolerate higher back-pressures were investigated, it became possible to determine the effect of higher flow-rates on dynamic capture efficiency.

At all flow-rates tested the dynamic capture efficiency for the cytokinins was essentially 100% (Table V). This is not an unexpected result if the binding of the hormone to antibody is considered to be instantaneous. (Since $K_a$ values of $10^{10}$ to $10^{13}$ $M^{-1}$ have been measured for these antibodies, this is a reasonable assumption.) With nearly instantaneous binding the limiting factor for solute retention is the availability of sufficient binding sites. The rate of lateral diffusion of molecules through the stationary mobile phase within the support determines the accessability of binding sites and therefore the effective capacity of the column.

Lateral diffusion of the solute to the antibody binding sites in the support is opposed by longitudinal movement (*i.e.* linear velocity) of the solute molecules in the column. Large molecule affinity separations are usually conducted at low flow-rates to overcome the limitation of slow lateral diffusion. Failure to allow sufficient mass

TABLE V

THE EFFECT OF FLOW-RATE ON DYNAMIC CAPTURE EFFICIENCY OF THREE IMMUNOSORBENTS

Standards of dihydrozeatin riboside (DHZR, 0.58 μg) or zeatin riboside (ZR, 0.5 μg), containing [$^3$H]DHZR or [$^3$H]ZR dialcohol respectively, were applied to columns at the flow-rates indicated. Dynamic capture efficiency was determined by scintillation counting of the radiolabeled standard not captured by the column during application of the sample and radiolabeled standard eluted from the column with methanol.

| *Flow-rate (ml/min)* | *FFS** | | *UEP*** | | *GPS**** | |
|---|---|---|---|---|---|---|
| | *Linear velocity (cm/min)* | *Capture efficiency (%)* | *Linear velocity (cm/min)* | *Capture efficiency (%)* | *Linear velocity (cm/min)* | *Capture efficiency (%)* |
| 1.0 | 1.6 | 100 | 5.9 | 100 | 1.6 | 100 |
| 1.5 | 2.5 | 97 | – | – | 2.4 | 100 |
| 2.0 | 3.3 | 100 | 11.8 | 99 | 3.2 | 98 |
| 2.5 | 4.1 | 96 | – | – | 4.1 | 98 |
| 3.0 | 4.9 | 97 | 17.6 | 98 | 4.9 | 98 |
| 4.0 | – | – | 23.3 | 97 | – | – |

| | *Column parameters* | | | |
|---|---|---|---|---|
| | *Bed height (cm)* | *Bed volume (ml)* | *Column diameter (cm)* | *Column capacity* |
| FFS | 3.3 | 2.0 | 0.9 | 2 μg ZR |
| UEP | 5.0 | 0.83 | 0.46 | 1 μg DHZR |
| GPS | 2.1 | 1.3 | 0.9 | 1 μg DHZR |

* FFS, Fast-Flow Sepharose (Pharmacia) with anti-ZR IgG linked by carbonyl diimidazole method.

** UEP, Ultraffinity-EP (Beckman) linked to anti-DHZR IgG.

*** GPS, Glycophase Silica (Diagnostic Specialties) linked to anti-DHZR.

transfer in the stationary mobile phase can result in peak splitting and elution of some solute in the exclusion volume[22]. This problem of lateral diffusion for large molecules is in striking contrast to the small molecule separations shown here, where lateral diffusion into the gel was clearly not a limiting factor.

Another factor affecting capture efficiency is the density of antibody immobilized on the support. In the case of small molecule immunosorption it is preferable to have the antibody density as high as possible. This is in contrast with the large molecule separations where multiple site attachment and steric problems are of major concern. The higher density of antibody is of benefit in small molecule isolations because it permits the use of smaller columns. This reduces the volume of eluent needed to elute the compounds from the column and minimizes the amount of non-specific background adsorption that can occur. Limiting the column volume also allows higher flow-rates if column cross-sectional area is kept constant, since column back-pressure is a function of column bed height.

With the exception of the Ultraffinity-EP HPLC column, higher flow-rates

could have been achieved through the immunosorbent columns with a low-pressure solvent delivery system. However, higher flow-rates than those used here (3.0 ml/min) may not be practical from a real world analysis standpoint. This flow study (Table V) was conducted with aqueous standards, but observations with plant tissue extracts indicated that practical flow-rate limits are reached when the matrix exerts a negative effect upon sample recovery (typically 10–20% reductions in recovery). Generally, reduction in recovery became evident at flow-rates between 3 and 4 ml/min. These flow-rate limitations are acceptable for most pre-column purification strategies which rely on gravity flow or low-pressure solvent delivery systems. In cases where high-performance separations are required, the use of HPLC columns such as the Ultraffinity-EP column may be practical.

*Seed extract analysis*

Cytokinins were isolated from young seeds using an Affi-Gel 10 anti-ZR column and analyzed by HPLC (Fig. 6A). Both ZR and DHZR were recovered from the soybean seed extract even though they were present at much lower levels than the unlabeled internal standards in the leaf sample. As with the leaf samples, the immunosorbent purification resulted in a sample with essentially no interfering substances when compared with the HPLC chromatogram of the crude plant extract (Fig. 6B). This example demonstrates the general applicability of the immunosorbent purification for different plant tissues and for trace enrichment of molecules which occur at relatively low concentrations.

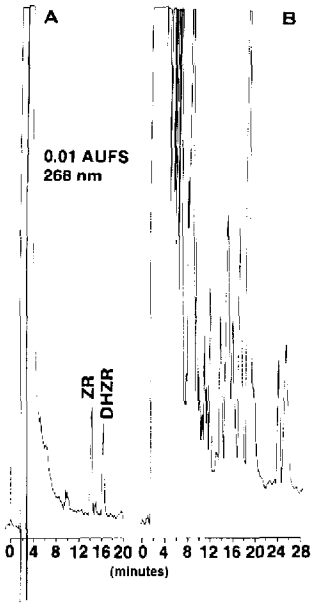


Fig. 6. Separation of zeatin riboside (ZR) and dihydrozeatin riboside (DHZR) from methanolic extract of 3.0 mm soybean embryos (40 mg fresh weight) by HPLC with anti-ZR CNBr-activated Sepharose 4B immunoaffinity purification (A), or no prior purification (B). Samples were separated on Ultrasphere ODS (5 $\mu$m, 25 cm × 0.46 cm I.D.) with 0.01 *M* acetic acid–0.005 *M* triethylamine (pH 3.35) at 1.5 ml/min (50°C) and detected by absorbance at 268 nm.

## CONCLUSIONS

The use of immunosorbents for trace analysis of plant hormones represents an advancement over purification procedures such as preparative HPLC, or solvent partitioning. Immunosorbents provide a less expensive, more rapid procedure than these traditional methods. Since the immunoaffinity supports are compatible with the crude plant extract, a minimum of sample preparation is required. This means that samples can be processed more quickly, with a minimum of manipulation, therefore reducing the chances of sample loss or degradation.

Immunosorbent isolations can be easily used with most analytical procedures. Since the purified antigen is eluted with an organic solvent (methanol), solvent reduction and preparation for subsequent analytical procedures is minimized. The cytokinin purifications described here have been used for preparation of plant extracts for gas chromatography–mass spectral analysis with a single purification step.

As with any immunoaffinity phenomenon, the effectiveness of immunosorption is ultimately limited by the nature of the antibody. The capture efficiency is a function of the concentration of the solute in solution, the $K_a$ of the antibody for the solute, and of the column capacity[3]. The characteristic cross-reactivity of the soluble antibody is largely unchanged on the immunosorbent. This characteristic can be used to advantage in the creation of immunosorbents with highly restricted specificity. Alternatively, the cross-reactivity of an antibody can be exploited to create group-specific immunosorbents which will capture a class of closely related molecules. Group-specific immunosorbents provide a much greater degree of purification than classical preparative separations and provide qualitative information about the purified solutes equivalent to the degree of specificity of the antibody. As demonstrated for the cytokinins ZR and DHZR, subsequent separation and analysis is nearly as simple as analysis of standards of the compounds of interest.

The use of immunoaffinity sorbents for cytokinins and a few other small molecules[4–10] clearly demonstrates the utility of this technique for sample purification. A wide variety of antibodies is already available which could be used to prepare immunosorbents for the purification of small molecules of interest in forensics, pharmaceuticals, environmental analysis, clinical and research medicine.

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