

Cytokinin binding proteins from mammalian sera

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Abstract. A cytokinin-binding protein fraction was isolated from normal rabbit sera by affinity chromatography. The protein fraction bound tritium labelled N⁶-(Δ^2 -risopentenyl) adenosine and the order of inhibition of this binding by competing non-radioactive compounds was, N⁶-(Δ^2 -isopentenyl) adenosine >N⁶-benzyladenosine >zeatin-riboside >N⁶-(Δ^2 -isopentenyl) adenine >kinetin riboside >adenosine. The protein fraction showed broad specificity, the preferred cytokinin being N⁶-(Δ^2 -isopentenyl) adenosine. This is the first report of the isolation of cytokinin binding proteins from mammalian sources.

Keywords. N⁶-(Δ^2 -isopentenyl) adenosine; binding protein; rabbit sera.

Introduction

Cytokinins promote cell division in plants (Miller, 1963; Letham, 1967). Cytokinins occur in plants in the free form and as components of their tRNAs. Receptor proteins for cytokinins and other phytohormones are present in plant tissues (Jayabaskaran, 1981; Dodds and Hall, 1980).

There is no evidence in literature that cytokinins occur free in mammalian systems or that they have any function in these systems. But cytokinins are known to bring about a variety of perturbations in mammalian cells. Thus, N⁶-(Δ^2 -isopentenyl) adenosine inhibits growth of leukaemic cells *in vivo* (Suk *et al.*, 1970) and *in vitro* (Rathbone *et al.*, 1972). Gallo *et al.* (1969) observed that N⁶-(Δ^2 -isopentenyl) adenosine inhibits mitosis of lymphocytes stimulated by phytohemagglutinin, at a concentration of 1 μ M. Quesney-Huneus *et al.* (1980) have shown that N⁶-(Δ^2 -isopentenyl) adenine and zeatin can replace mevalonate to restore DNA replication in compactin blocked baby hamster kidney cells, but that N⁶-(Δ^2 -isopentenyl)adenosine is not effective.

In animal systems, cytokinins have been detected only in tRNAs (Letham, 1978; Senapathy, 1978). It is known that the presence of N⁶-(Δ^2 -isopentenyl)adenosine in certain species of tRNAs is essential for the proper function of those tRNAs in

Abbreviations used: i⁶A/i⁶ Ado, N⁶-(Δ^2 -isopentenyl) adenosine; pi⁶Ap, N⁶-(Δ^2 -isopentenyl) adenosine 5',3'(2') diphosphate; pi⁶Ap>, 5'-phosphoryl-N⁶-(Δ^2 -isopentenyl) adenosine 3'-2' cyclic phosphate; EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; AH-Sepharose, Aminohexyl Sepharose; TBS, Tris-buffered saline (0.01 M Tris-HCl, pH 7.5 containing 0.14 M NaCl and 0.02% NaN₃); TBHS, Tris-buffered high concentration saline (0.01 M Tris-HCl, pH 7.5 containing 0.64 M NaCl and 0.02% NaN₃).

protein synthesis, probably in their binding to ribosomes. In a preliminary communication Fox and Erion (1975) have reported the presence of cytokinin binding sites on rat liver ribosomes.

When N^6 -(Δ^2 -isopentenyl)adenosine antibodies were purified in our laboratory on an AH-Sepharose- pi^6A_p column or an i^6Ado -AH-Sepharose column (Senapathy, 1978; Jayabaskaran, 1981) and the purified antibodies were subjected to gel electrophoresis, two protein bands, in addition to IgG, were observed. It was suspected that these could be N^6 -(Δ^2 -isopentenyl) adenosine-binding proteins normally present in rabbit sera, and attempts were made to isolate them. This paper reports the affinity chromatographic isolation of a cytokinin binding protein fraction from normal rabbit sera.

Materials and methods

Serum was prepared from rabbits obtained from the local market. N^6 -(Δ^2 -isopentenyl)adenosine, N^6 -(Δ^2 -isopentenyl)adenine, zeatinriboside, N^6 -benzyladenosine, kinetinriboside, adenosine, guanosine, mevalonic acid, AH-Sepharose and bovine serum albumin were from Sigma Chemical Co., St. Louis, Missouri, USA. Nitrocellulose filters (0.45 μ , MDI filters) were from microdevices, Ambala. Tritium labelled N^6 -(Δ^2 -isopentenyl)adenosine was prepared at the Bhabha Atomic Research Center, Bombay, by the Wilzbach procedure (1957), by exposing N^6 -(Δ^2 -isopentenyl)adenosine to tritium gas. It was purified by chromatography on Sephadex LH-20 (Armstrong *et al.*, 1969). The specific activity of the 3H - i^6A was 56,275 cpm/pmol.

Preparation of N^6 -(Δ^2 -isopentenyl) adenosine 5',3'(2') diphosphate

The synthesis was done by the phosphorylation of N^6 -(Δ^2 -isopentenyl) adenosine with cyanoethyl phosphate in the presence of dicyclohexyl carbodiimide and the subsequent removal of the cyanoethyl group under alkaline conditions, according to the procedure of Humayun (1974).

Preparation of the affinity column

The 5'-phosphate group of N^6 -(Δ^2 -isopentenyl)adenosine 5'3'(2') diphosphate was coupled to the amino groups in AH-Sepharose using EDC as the condensing agent, according to the procedure used by Humayun (1974) for coupling the same diphosphate to bovine serum albumin. Humayun has shown that on EDC treatment the 5'3'(2') diphosphate cyclises to give 2'-3' cyclic phosphate leaving the 5' phosphate for condensation to the amino groups. The amount of pi^6Ap coupled to AH-Sepharose was spectroscopically determined after solubilization by NaOH and sodium borohydride treatments (Failla and Santi, 1973) and was found to be 2.7 μ mol per ml of packed volume of AH-Sepharose.

Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

For analyzing the specificity of the binding of 3H - i^6A to the isolated protein fraction, the nitrocellulose filter assay was carried out as described by Humayun and Jacob (1973) for antibody and hapten systems.

Results

Affinity chromatography

AH-Sepharose- i^6A column (0.4×3.5 cm) was equilibrated with TBHS (0.01M Tris-HCl, pH 7.5 containing 0.64M NaCl). Fifty ml of normal rabbit sera was made up to 150 ml with concentrated buffer so that the final buffer composition was the same as that of the equilibrating buffer and then loaded onto the column at a flow rate of 3 ml/h. The column was first washed with TBHS (200 ml) and then eluted with 5mM i^6A in TBHS. One ml fractions were collected and dialysed separately against TBS (0.01M Tris-HCl, pH 7.5 containing 0.14M NaCl). Protein in each fraction was estimated by Lowry's method. The elution profile is shown in figure 1. There is only one protein peak (895 μ g). Fractions were pooled, concentrated and dialysed extensively in TBS to remove i^6A .

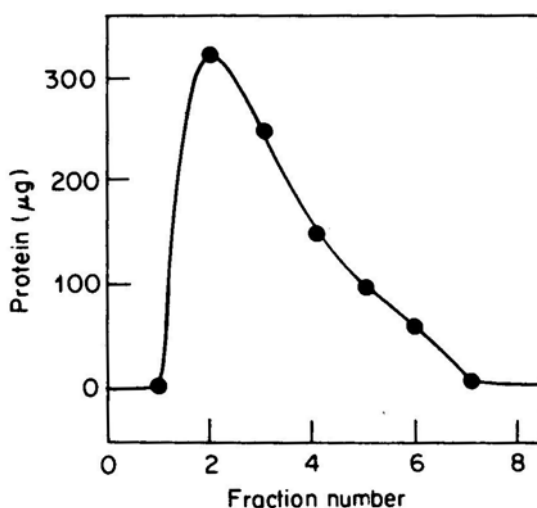


Figure 1. Affinity chromatography for the isolation of cytokinin binding protein from normal rabbit serum.

Removal of bound i^6A

The dialysed protein fraction (400 μ g) was treated with dextran coated charcoal (0.5% charcoal and 0.05% dextran T. 70 in TBS) for 24 hours then the charcoal was removed by centrifugation (Muniyappa and Adiga, 1980).

Assay for the binding of $^3H-i^6A$ by gel filtration

The dextran coated charcoal treated protein fraction was incubated with $^3H-i^6A$ chromatographed on Sephadex G-25 and the radioactivity monitored, bovine serum albumin and protein untreated with dextran coated charcoal were also incubated with $^3H-i^6A$ and chromatographed separately on the same column as controls. Two peaks were noticed, one at void volume due to protein-bound $^3H-$

i^6A and another due to free $^3H-i^6A$ region. Table 1 shows the radioactivity in the first peak in the experiments. The results show that $^3H-i^6A$ bound to the protein fraction only when bound i^6A was removed by dextran coated charcoal treatment.

Table 1. $^3H-i^6A$ binding by chromatography on Sephadex G-25.

Sample	$^3H-i^6A$ bound* (cpm)
Bovine serum albumin (BSA) (50 μ g)	580
BSA (50 μ g)+dialysed protein (2 μ g)	631
BSA (50 μ g)+dialysed and dextran coated charcoal treated protein (2 μ g)	4027

* The assay mixture (0.4 ml in TBS) containing $^3H-i^6A$ (56,275 cpm) and the sample was incubated at 28°C for 2 h and applied on Sephadex G-25 (1 \times 13 cm) and eluted with TBS. After background subtraction, counts in the fraction around void volume were taken at $^3H-i^6A$ bound to protein.

The protein was incubated with $^3H-i^6A$ and chromatographed on Sephadex G-200 (figure 2). Protein-bound $^3H-i^6A$ was eluted at the void volume indicating a molecular weight above 200,000 for the protein ligand complex.

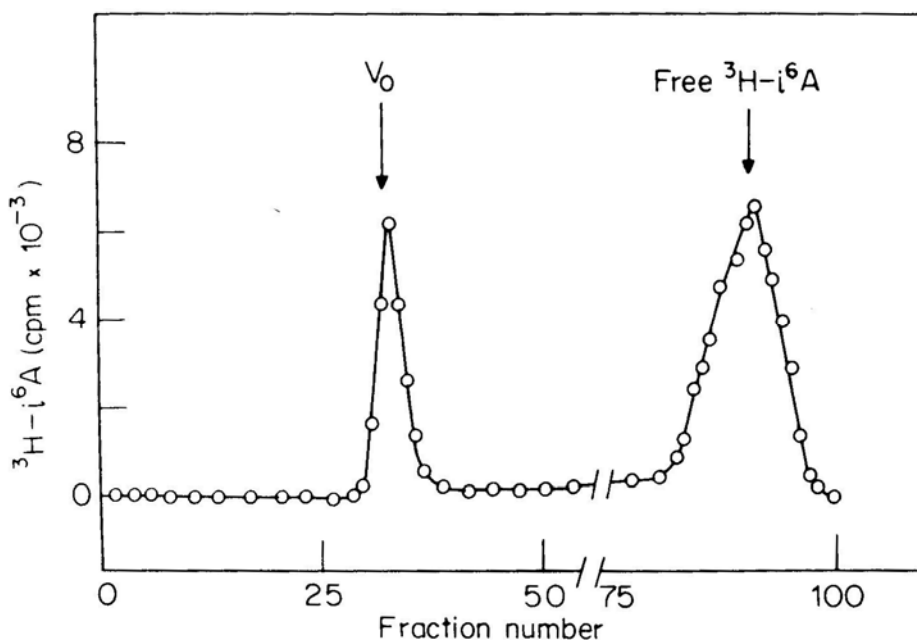


Figure 2. $^3H-i^6A$ binding by gel filtration.

The reaction mixture (0.4 ml in TBS) containing $^3H-i^6A$ (1125,500 cpm), the protein (6 μ g) and bovine serum albumin (150 μ g) was incubated at 28°C for 2 h and applied onto a Sephadex G-200 column (2 \times 50 cm) pre-equilibrated with TBS and eluted with TBS at a flow rate of 2 ml/h. Fractions of 2 ml were collected and 0.6 ml aliquots spotted on Whatman No. 3 paper. After drying, the papers were counted in a scintillation counter after addition of 5 ml of scintillation fluid.

Specificity of the binding of $^3\text{H-i}^6\text{A}$ by nitrocellulose filter assay

The protein fraction was found to bind to $^3\text{H-i}^6\text{A}$ by the nitrocellulose filter assay. The specificity of this binding was determined by measuring its inhibition by non-radioactive cytokinins and related compounds (table 2). The order of inhibition was N^6 -(Δ^2 -isopentenyl) adenosine > N^6 -benzyladenosine > zeatinriboside > N^6 -(Δ^2 -isopentenyl) adenine > kinetinriboside > adenosine; mevalonic acid and guanosine do not inhibit the binding.

Table 2. Binding of $^3\text{H-i}^6\text{A}$ to the protein fraction in the presence of unlabelled compounds.

Unlabelled competitors	Bound $^3\text{H-i}^6\text{A}$ (% of control)
None (control)	100.0
N^6 -(Δ^2 -isopentenyl) adenosine	12.6
N^6 -(Δ^2 -isopentenyl) adenine	53.9
N^6 -benzyladenosine	32.6
Zeatinriboside	42.1
Kinetinriboside	67.6
Adenosine	68.7
Guanosine	100.0
Mevalonic acid	100.0

The control assay mixture in a total volume of 0.4 ml in TBS consisted of 2 μg of dextran coated charcoal treated protein fraction, 50 μg of bovine serum albumin, 56,275 cpm of $^3\text{H-i}^6\text{A}$, whereas the experiments contained in addition $2.5 \times 10^{-6}\text{M}$ concentration of the non-radioactive competitor also. Incubation was at 28°C for 2 h and then protein bound radioactivity was determined by nitrocellulose filter assay. Control (no inhibitor) bound had 5536 cpm and 50 μg of bovine serum albumin retained 1862 cpm.

Discussion

About 900 μg of a protein fraction has been isolated from 50 ml of normal rabbit serum by affinity chromatography on AH-Sepharose- pi^6Ap column, using N^6 -(Δ^2 -isopentenyl) adenosine for elution (figure 1). This protein fraction was present in all the 8 rabbits so far tested. This protein fraction bound tritiated N^6 -(Δ^2 -isopentenyl) adenosine when the bound non-radioactive N^6 -(Δ^2 -isopentenyl) adenosine was removed using dextran coated charcoal (table 1). When tritiated N^6 -(Δ^2 -isopentenyl) adenosine and the protein fraction were chromatographed on Sephadex G-200, $^3\text{H-i}^6\text{A}$ bound-protein eluted at the void volume indicating a molecular weight above 200,000 for the protein ligand complex (figure 2). The specificity of the protein fraction when tested by competition experiments in its binding to tritiated N^6 -(Δ^2 -isopentenyl) adenosine, showed selectivity to cytokinins in general, N^6 -(Δ^2 -isopentenyl) adenosine being the most preferred one (table 2). Polyacrylamide gel (7%, pH 8.6) electrophoresis of the protein fraction (data not shown) gave two bands, one at the region of the IgG marker and another at the region of the bovine serum albumin marker. $^3\text{H-i}^6\text{A}$ binding activity demonstrated for the protein fraction by gel filtration on Sephadex G-200 is likely to be due to the slow moving band. The conditions used for the removal of the bound ligand from the proteins, might not have succeeded in removing the bound i^6A from the protein

corresponding to the faster moving band or they might have denatured the protein. Further studies are needed to clarify these points.

Recently a cytokinin binding protein fraction has been isolated in our laboratory from goat sera (unpublished results of M. Viswanathan and T. M. Jacob).

The discovery that normal mammalian sera contain cytokinin binding proteins, is of considerable importance. It gives an indication that N⁶-(Δ^2 -isopentenyl) adenosine may have some normal functions in mammalian systems, probably mediated by the binding proteins. It is expected that the cytokinin-binding proteins isolated will serve as tools to understand these functions. It is possible that cytokinins may be involved in the control of cell proliferation at least in some mammalian cells for e.g. lymphocytes.

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