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Glucosyl Zeatin and Glucosyl Ribosylzeatin from *Vinca rosea* L. Crown Gall Tumor Tissue[1]

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ABSTRACT

Recently detected but unidentified cytokinin activity in crown gall tumor tissue from *Vinca rosea* L. grown on media containing sources of reduced nitrogen has now been attributed to two adenine-type cytokinins. These compounds are glucopyranosyl derivatives of zeatin and ribosylzeatin. The substitution in each case is on the isopentenyl chain of the parent compound. Neither of these compounds had activity in the soybean callus bioassay at concentrations lower than 1 nM whereas zeatin had activity at 0.1 nM.

We have previously reported that crown gall tumor tissue from *Vinca rosea* L. accumulates significantly higher amounts of extractable cytokinins when cultured on media supplemented with various sources of reduced nitrogen than when cultured on the control medium (20). With 3.8 mM ammonium chloride as the reduced nitrogen additive, the cytokinins zeatin and ribosylzeatin account for over half of the increase in biological activity. Approximately one-third of the increase is associated with an active fraction which we had not previously detected in this tissue cultured on the the basal medium. Although the chromatographic behavior of this fraction resembled that of known glucosyl derivatives of zeatin (6, 16-19, 25), further purification and study were necessary for definite identification.

Such purification has now been achieved, and it is clear that the additional activity is due to two factors. The compounds are glucosyl derivatives of zeatin and ribosylzeatin, with the glucose in each case being a glucopyranosyl modification of the hydroxyl group on the isopentenyl chain of the parent compound. The purification and identification of these factors are described herein.

MATERIALS AND METHODS

Tissues and Bioassays. A start of the *V. rosea* tumor tissue (A6 line) was kindly supplied by H. Wood of The Rockefeller University. Tissues used for extraction purposes were cultured on a modified White's medium supplemented with 3.8 mM NH_4Cl (20). Inoculations were made from tissues grown on the medium without ammonium chloride. The soybean callus tissue bioassays for cytokinin activity were carried out as previously described (12).

Chromatography. Chromatography was performed in an ascending fashion on Whatman No. 1 filter paper or, when noted, on 1-mm-thick layers of silica gel (Merck PF-254). Solvent systems used were (v/v): *sec*-butyl alcohol saturated with water; *tert*-butyl alcohol-water (3:2); water; 1-butanol-NH_4OH-water (86:5:14); chloroform-methanol (9:1); and 0.03 M borate (pH 8.4).

Column chromatography was performed on PVP (4) and LH-20 (1) as described below. The PVP and LH-20 were obtained from Calbiochem and Pharmacia, respectively.

Enzyme Studies. β-Glucosidase (emulsin) from almond (Mann Research Laboratories) was used to test the possibility that the active compounds are glucosides. Purified cytokinins were incubated with the enzyme for 2 hr at 30 C. The reaction mixture consisted of 0.1 ml substrate, 0.1 ml sodium acetate (0.05 M, pH 5.15), and 0.3 ml enzyme (135 μg/ml). The enzyme was also used for detecting cytokinin glucosides in crude tissue extracts. In such an experiment, 10 g of 7-week-old tumor tissue which had been grown on the ammonium-supplemented medium was shaken in sufficient ethanol to bring the final concentration to 80% and the mixture was centrifuged. The extract was chromatographed with water-saturated *sec*-butyl alcohol. The sections from R_f 0.35 to 0.6 which contained the activity under investigation (20) were eluted with methanol. The methanol was removed with an air stream and the resulting aqueous layer was treated with β-glucosidase as described above. The products of the reaction were rechromatographed with the *sec*-butyl alcohol system and the sections from R_f 0.7 to 1.0, which contain the areas to which zeatin and ribosylzeatin run, were eluted with 95% ethanol. The eluates were then chromatographed with the borate solution and the resulting chromatogram sectioned and bioassayed.

Yeast hexokinase (A grade) and glucose-6-P dehydrogenase (A grade) used for the identification of glucose were obtained from Calbiochem. Coupled incubations were run at room temperature. The reaction mixture contained 0.5 ml glycylglycine (0.25 M, pH 8), 0.1 ml NADP (0.01 M), 0.2 ml $MgCl_2$ (0.15 M), 0.1 ml ATP (dipotasssium salt, 0.01 M), 0.4 ml glucose-6-P dehydrogenase (0.1 mg/ml), 0.4 ml hexokinase (0.1 mg/ml), and 0.5 ml substrate. Reduction of NADP was followed at 340 nm. The specificity of the reaction was demonstrated by its reactivity with D-glucose but not with D-fructose or D-mannose (7, 9) (see Table II).

Nucleosidase from *Lactobacillus plantarum* was prepared as described by Wang (24) and used for detecting ribose on the cytokinins (14). The reaction mixture consisting of 2 volumes of 0.05 M tris (pH 7.5) and 3 volumes of enzyme preparation was added to the dried (*in vacuo*) cytokinin. Incubations were run at 37 C. A changed R_f value of the cytokinin indicated the presence of ribose.

Purification of Cell Division Factors. One thousand g of 7-week-old tumor tissue (frozen) were added to 1,000 ml of cold ethyl acetate. The tissue thawed with frequent, vigorous stirring

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of the mixture. After thawing was completed and the tissues extracted, the ethyl acetate was removed and the tissue was then further extracted with seven successive, 333-ml volumes of cold ethyl acetate. The ethyl acetate extracts were discarded. The tissue was next extracted with seven successive, 333-ml volumes of cold *sec*-butyl alcohol. This fluid was reduced under an air stream to a volume of approximately 30 ml. Extraction of this highly viscous layer with 95% ethanol and then small volumes of 80% ethanol left a white precipitate. The combined ethanolic extracts were reduced to an aqueous layer under an air stream and centrifuged to remove materials which floated and appeared to be lipids. The solution was made 35% with respect to ethanol and applied in a volume of 50 ml to an LH-20 column (4 × 50 cm) which was developed with 35% ethanol. The column was run at a flow rate of 100 ml/hr and 9-ml fractions were collected. Fractions 52 through 85, which contained the activities being studied, were pooled, reduced to an aqueous volume of 35 ml, and applied to a PVP column (4 × 30 cm). The column was developed with water at a flow rate of 96 ml/hr and 6-ml fractions were collected. Individual fractions with biological activity were chromatographed on paper first with the *sec*-butyl alcohol solvent, next with the *tert*-butyl alcohol system, and then with water.

Potassium Permanganate Oxidations. Approximately 0.06 μmol of cytokinin sample was dried *in vacuo*. One ml of 0.01% $KMnO_4$ (chilled) was added and allowed to react for 10 min. The reactions were stopped by the addition of an excess of chilled 95% ethanol.

RESULTS

Treatment of Chromatographed Crude Extracts with β-Glucosidase. The extractable cytokinin activity which runs to an R_f of approximately 0.47 with the *sec*-butyl alcohol system (20) was examined to determine if the activity could be due to one or more glucosyl derivatives of known cytokinins. Crude tissue extracts (80% ethanol) were subjected to chromatography and β-glucosidase as described under "Materials and Methods." With the final chromatographic system (borate), two peaks of biological activity were observed. These peaks had R_f values of approximately 0.48 and 0.82 which corresponded to those of synthetic zeatin and ribosylzeatin.

Identification of Cytokinin Glucosides. From the PVP effluent in the purification procedure, two peaks of biological activity were detected. The first (termed factor I) eluted in fractions 70 to 83 and ran with R_f values of 0.42, 0.82, and 0.70 in the *sec*-butyl alcohol, *tert*-butyl alcohol and water systems used in the final purification steps. The second factor (termed factor II) eluted in fractions 84 to 96 and ran with R_f values of 0.54, 0.80, and 0.58, respectively, in the same systems. Based on their relationship to ribosylzeatin and zeatin (see following identifications), assumed molar extinction coefficients at 267 nm (methanol) of 19,000 for factor I and 18,000 for factor II gave extraction yields of 0.53 and 0.58 μmol, respectively, per kg of tissue.

The UV spectral data for factor I are presented in Table I. The absorption peaks of this factor are very similar to those reported for ribosylzeatin (11). Treatment of I with β-glucosidase yielded a compound which, in the *sec*-butyl alcohol system, ran with synthetic ribosylzeatin (R_f 0.74). The UV spectrum in methanol and the mass spectrum (15) of the product were those of ribosylzeatin. Hydrolysis of I with 0.5 N HCl for 1 hr at 100 C liberated a factor which ran with zeatin in the *sec*-butyl alcohol (R_f 0.9) and water (R_f 0.53) systems and which had the UV absorption spectrum of zeatin in methanol. Hydrolysis of a sample of synthetic ribosylzeatin in the same fashion liberated a product with the same properties as the product of the hydrolysis of I. Further examination of the supposed zeatin from the hydrolysis of I showed that it ran with synthetic zeatin (R_f 0.32) and not with its *cis*-isomer (R_f 0.39) on silica gel plates developed with the chloroform-methanol system which distinguishes between the *cis*- and *trans*-isomers of ribosylzeatin (21). Factor I, therefore, contains zeatin. The presence of glucose on the chromatogram (R_f 0.30–0.36, *sec*-butyl alcohol system) as a product of the acid hydrolysis was demonstrated with a coupled enzyme assay using hexokinase and glucose-6-P dehydrogenase (Table II).

Table I. Absorption Peaks of Factors I and II from Vinca rosea Tumor Tissue and of the Product Obtained by Action of Ribonucleosidase on Factor I

Solvent: Methanol		0.1 N HCl (nm)	0.1 N NaOH
Factor I	267, 211	264, 205	267, 217
Factor II	267, 209	273, 205	274*, 216
Product	268	272	274*

*Also a shoulder at 283

Table II. Detection of D-Glucose in Acid Hydrolysates of Factors I and II

A glucose-6-phosphate dehydrogenase couple was used for detection. An absorbancy change of 1.0 at 340 nm corresponds to the complete reaction of 6.8 μg D-glucose. Reactions were complete in 20 min.

Material Tested	ΔA_{340} nm
7.2 μg D-Glucose	0.099
7.5 μg D-Fructose	-0.004
7.5 μg D-Mannose	0.000
7.2 μg D-Glucose, minus ATP	-0.001
7.2 μg D-Glucose, minus NADP	0.000
Eluted glucose positions from chromatograms of acid hydrolysates of:	
Synthetic ribosylzeatin	-0.001
Factor I	0.032
Synthetic zeatin	-0.007
Factor II	0.032
D-Glucose (14.4 μg applied to chromatogram)	0.046

Factor I, from the above data, appeared to be a glucoside derivative of ribosylzeatin, substituted either on the ribose moiety or the isopentenyl chain. A crude enzyme preparation (ribonucleosidase) from *L. plantarum* which has been shown to remove ribose from a variety of N^6-substituted adenosines (14) was employed to locate the glucose. Factor I (0.088 μmol) was incubated for 1 hr with the enzyme reaction mixture. Several products resulted, one of which had R_f values normally seen for factor II in the *sec*-butyl alcohol (0.48), water (0.63), and 1-butanol-ammonia systems. The recovered product had the spectral characteristics of factor II (Table I). Incubation of synthetic zeatin (0.088 μmol) and D-glucose (1 μmol) with the nucleosidase did not result in the appearance of this factor. The glucose thus appeared to be attached to the isopentenyl chain of ribosylzeatin as was determined for factor II (see following identification). The mass spectral studies by Morris (15) on the isolated material led to the same conclusion.

Further strong evidence for the identity of factor I was provided by a potassium permanganate oxidation. $KMnO_4$ attacks the double bond in the isopentenyl group of adenine-type cytokinins resulting in the complete loss of the side chain or cleavage at the double bond (10, 13). Oxidation of factor I should, therefore, yield the same products as oxidized ribosylzeatin if the glucose is attached at a terminal carbon of the isopentenyl group. Oxidation of I yielded two products detected by chromatography

(the 1-butanol ammonia system). One ran with adenosine (R_f 0.20) and a product of the oxidation of ribosylzeatin. The other (R_f 0.04) ran with a second product of the ribosylzeatin oxidation. In further chromatography with water, the supposed adenosine products of the above reactions ran with synthetic adenosine (R_f 0.53) and were cleanly separated from synthetic adenine (R_f 0.39). These data also, therefore, are consistent with the conclusion that factor I is ribosylzeatin substituted on the isopentenyl chain.

Factor II was identified in the same manner as factor I. The UV spectral data for the purified factor are presented in Table I. The peaks are similar to those reported for zeatin (8, 22) and zeatin substituted with glucose on the isopentenyl chain (6, 18). β-Glucosidase treatment yielded a compound which ran with zeatin (R_f 0.84) with the *sec*-butyl alcohol system. The UV spectrum in methanol of the liberated compound showed the 267 nm zeatin peak, and the mass spectral data (15) confirmed its zeatin identity. Acid hydrolysis of factor II yielded products which were identical in all tests with two products (zeatin and D-glucose [Table II])of the hydrolysis of I. Factor II, therefore, appeared to be zeatin substituted with a glucose moiety. The UV and β-glucosidase data were consistent with a substitution on the iospentenyl chain (6, 18). Comparisons of these data with similar data on synthetic cytokinin glucosides of benzyladenine (3) also supported this contention. Mass spectral studies by Morris (15) on the intact molecule resulted in the same conclusion.

As with factor I, oxidation with potassium permanganate was used to confirm the identity of factor II strongly. Oxidation of II yielded a product that ran with adenine (R_f 0.21) and a product of a zeatin oxidation with the 1-butanol-ammonia solvent system. Factor II also yielded small amounts of a second, unidentified product (R_f 0.09 in the same system). When the chromatographic mobilities of the supposed adenine products from the above reactions were compared with synthetic adenine and adenosine with water, they ran with the adenine (R_f 0.39) and not with adenosine (R_f 0.53). These results are consistent with the identification of factor II as zeatin substituted on the isopentenyl chain.

From the above data and those of Morris (15), factor II was assigned the structure N^6-(4-D-glucopyranosyl-3-methyl-2-*trans*-butenyl)aminopurine. Factor I is the corresponding ribonucleoside derivative. The β-glucosidase data are consistent with a β configuration.

Both glucosyl compounds were compared with synthetic zeatin over a range of concentrations in the soybean callus tissue bioassay. Whereas zeatin had threshold activity at 0.1 nM, neither of the glucosyl derivatives had activity detectable below 1 nM.

DISCUSSION

The identities of two factors which account for a substantial portion of the cytokinin activity in *V. rosea* crown gall tumor tissue cultured on media containing ammonium chloride have been established. These factors are glucosides of zeatin and ribosylzeatin, substituted at the hydroxyl position of the isopentenyl chains. Extensive mass spectral studies by Morris (15) on the factors supplied by this laboratory have led to the same conclusions. Glucosides of zeatin with the glucose on the side chain or on the 7 or 9 positions have been previously detected in plant tissues (6, 18, 25). The glucosyl ribosylzeatin, to the best of our knowledge, has not been previously identified in any plant system. Either one or both of these compounds may account for an unidentified cytokinin activity from crown gall of *Parthenocissus tricuspidata* (23) which had similar chromatographic properties.

We do not know the function of these factors. They are present in detectable quantities only when this tissue is grown on a medium containing a reduced nitrogen supplement, a condition that markedly enhances the amounts of extractable zeatin and ribosylzeatin (20). The ideas have been advanced that glucose derivatives are storage (2, 5, 16) or detoxification (2) products of cytokinins, although the latter authors argued for the storage function. The results obtained here are consistent with either idea, as in either case the glucosylation might occur in response to relatively high levels of the parent cytokinins.

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