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First Evidence for Polyamine Conjugation Mediated by an Enzymic Activity in Plants[1]

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ABSTRACT

An enzyme activity, found for the first time in plants, mainly located in the 22,000*g* supernatant of the crude extract of sprout apices of *Helianthus tuberosus* L. cv OB1 tubers, is able *in vitro* to covalently bind polyamines to endogenous substrates of different molecular weights. The major assay parameters, such as pH, dithiothreitol, and extract concentrations were optimized. The time course of the reaction, the dependence on putrescine concentration, its competition with histamine, the capacity to bind spermidine and spermine better than putrescine, the stability of the reaction product and analysis of the latter by sodium dodecyl sulfate polyacrylamide gel electrophoresis and thin-layer chromatography suggest that putrescine is linked to endogenous substrates by means of an enzyme reaction that shows some similarities with transglutaminase activities detected in animals. However, the activities of the crude extract and of a fraction eluted from a Sephadex G25 column were not affected by $CaCl_2$ concentrations lower or equal to 5 millimolar; 4 or 10 millimolar EGTA caused a very small reduction; higher concentrations of $CaCl_2$ and 15 millimolar or more of EDTA were inhibitory. *N,N'*-dimethylcasein was not recognized as a substrate. These data indicate that this activity also displays some characteristics which are different from those of animal transglutaminases.

One of the reasons for the increasing importance assumed by spermine, spermidine, and putrescine in biology is the role that they play in modulating some enzyme activities (proteolysis, protein phosphorylation, ornithine decarboxylation) or in interacting with structural proteins (actin, myosin, fibrin and fibronectin, tubulin) (2, 24, 26, 27). Polyamines, owing to their chemical nature, can form hydrogen, ionic, or covalent linkages with other molecules. In some cases, polyamines linked to particular proteins have been isolated (1, 2, 7, 11, 13, 23, 26, 27). In animals, posttranslational covalent linkages of polyamines to numerous proteins have been demonstrated. They are solely catalyzed by transglutaminases (R-glutaminyl-peptide: amine-γ-glutamyl-transferase, EC 2.3.2.13) and form cross-linked and noncross-linked complexes with two or one peptide-bound glutamine residues respectively (12, 13, 28, 33; reviewed in Refs. 11 and 17). In plants, although various other types of conjugates (*e.g.* with hydroxycinnamic acids) have been detected (21), these covalently bound polyamine-protein complexes are practically unknown. However, in activated parenchyma slices of *Helianthus tuberosus* tubers, bound polyamines seem to be mainly linked to proteins (31), and we had preliminary data suggesting the existence of a transglutaminase-like activity (22). Moreover, when labeled putrescine was added to the culture medium, radioactive complexes, extracted in particular phases of the cell cycle from these synchronously growing cells, have been detected in various SDS-gel electrophoretic bands (22).

In the present study, we examined the possibility that also in plants an enzyme activity, perhaps a transglutaminase activity, might catalyze the formation of such complexes. We used the shoot apices of *Helianthus* sprouts, where many meristematic cells divide actively, but asynchronously; therefore, if this enzyme activity is present and expressed in certain phases of the cell cycle, it should be easily detected.

MATERIALS AND METHODS

Plant Material and Enzyme Assay. The apices (3–4 mm of the apical region) of the sprouts growing from tubers of *Helianthus tuberosus* L. cv OB1 were sterilized with sodium hypochlorite (6% active Cl^-) for 15 min and used fresh or after storage at −80°C. Apices were rapidly ground in an ice-cold mortar with two volumes per g fresh weight of 50 mM Tris-HCl buffer (pH 8) containing 45 mM freshly prepared DTT. After filtration through four layers of cheesecloth and avoiding as much as possible contact with air, one part of the extract was used for the enzyme assay, performed with some modification according to Haddox and Russell (13). The other part was precipitated with 10% TCA (w/v) for protein determination. One hundred μl of the crude extract (containing more than 0.4 mg protein) or its 22,000*g* supernatant (or pellet) were added to 100 μl of 150 mM Tris-HCl (pH 8.2), 50 μl of either *N,N'*-dimethylcasein (1.5 or 30 mg ml^{-1} in 50 mM Tris-HCl, pH 8) or of 50 mM Tris-HCl (pH 8), and 50 μl of 1.2 mM putrescine labeled with 1,480 kBq ml^{-1} of [1,4(*n*)-^{3}H]putrescine (833 TBq mol^{-1}, unless otherwise indicated) (Amersham, UK). This 300 μl assay mixture, having a pH of 7.8, was incubated at 30°C for 30 min, was placed on Whatman No. 3 MM filter paper discs after stopping the reaction with 20% TCA (w/v), and then was filtered using a multifilter apparatus (Millipore) and repeatedly washed with 5% TCA also containing 0.2 M KCl. The dried filters were cut into small pieces, put in vials containing 4.5 ml of scintillation cocktail (MP, Beckman) and counted for 20 min in a Beckman LS 1800 counter.

Other extraction media were also tested, but were less effective: 0.25 M sucrose and Tris buffer containing different molarities of DTT.

Other experiments were performed with 92.5 kBq per assay of [1,4-^{14}C]putrescine or [1,4-^{14}C]spermidine or [1,4-^{14}C]spermine (all having a specific activity of 4.37 TBq mol^{-1}), (Amersham, UK). The effects of 5 mM histamine, or various concentrations of $CaCl_2$, EDTA, EGTA, DTT, or ion deprivation by elution on a Sephadex G25 column were also tested as well as different amounts of plant extract, *N,N'*-dimethylcasein, and [^{3}H]putrescine.

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To evaluate the formation of other types of linkages (ionic or hydrogen bonds), at the end of the assay, 3 ml 20% TCA were added to the mixture, which was then filtered, treated with 9 ml 1 M NaCl, and washed as described; alternatively, NaCl was added directly to the assay mixture, which was then filtered and the filters were treated with 20% TCA and washed. In another experiment, the reaction product treated with 20% TCA was filtered and washed with 30 ml of 5% TCA plus 0.2 M KCl also containing 5 mM putrescine.

All the reagents, unless otherwise indicated, were purchased from Sigma. Each experiment was repeated at least twice with different extracts, with 3 to 6 replicates for each assay. The data are expressed as pmol [^{3}H]putrescine (mg protein)$^{-1}$ 30 min^{-1} where proteins are only the endogenous ones (casein through present was not considered). The isotopic dilution is reported in the legends of tables and figures. The physiological stage of the sprouts influenced the binding capacity of the extracts. Therefore, the data from separate experiments may be somewhat different.

Polyamine Determination. Free polyamines in crude extracts of the sprout apices were detected after dansylation (32) in the 5% TCA supernatant after centrifugation (3000 rpm, 10 min). Polyamines were separated on TLC plates with a concentrating zone (Merck). Mono- or bi-dimensional chromatographies were eluted in cyclohexane:ethylacetate (3:2, v/v) and/or in chloroform:triethylamine (5:1, v/v). Fluorescence was measured in scraped spots extracted with 1 ml of anhydrous acetone. The conjugated polyamines of the assay product collected on filters or precipitated twice with 10% TCA were either separated on SDS-PAGE or hydrolyzed overnight with 6 N HCl at 120°C. The hydrolysate was taken to dryness, resuspended in 5% TCA, brought to pH 8.5, dansylated, and chromatographed as described. Radioactivity in the scraped spots was measured after fluorescence decay.

Protein Determination. Proteins in the crude extract (or in the 22,000*g* supernatant and pellet) were measured on the TCA precipitate, solubilized in 1 N NaOH, using the Bradford (6) method (Bio-Rad Protein Assay) because DTT did not interfere with this assay, as it did with the Lowry method (20).

SDS-PAGE. Assay products with labeled polyamines were separated by gel electrophoresis with sodium dodecyl sulphate (16). The entire lanes were cut into either Coomassie positive or negative bands, and their radioactivity was counted after gel dissolution.

Calcium Determination. Total calcium content in the crude homogenate and 22,000*g* supernatant was measured by atomic absorbance spectrophotometry (Varian S 11 spectrophotometer).

RESULTS AND DISCUSSION

Definition of the assay conditions. In order to optimize the assay conditions, various parameters were checked and some modifications of the method normally used with animal extracts were adopted. It is known that oxidases are very active in *Helianthus tuberosus* (3). In fact, the concentration of DTT in the extraction buffer and in the assay mixture was critical to prevent inactivation of the enzyme activity (Table I). If, in addition to the concentrations reported in Table I, the same 10 or 30 mM concentration of DTT was present in the crude extract as well as in the assay mixture, the reaction was blocked (data not shown). The polyamine-binding activity, recently found in the storage parenchyma of *H. tuberosus* tubers, could proceed in the presence of much lower concentrations of DTT (2 mM) (30). In animal systems, DTT is used, if at all, at similarly low concentrations in the assay mixture (13, 15, 23).

The optimum pH was 7.8 at 30°C, and a similar pH has been used for tranglutaminase assays in animals (4, 10, 13). *N,N'*-dimethylcasein, normally used as the exogenous acyl donor substrate when assaying animal tranglutaminases, did not influence the activity of this plant enzyme at low concentrations. On the contrary, at high concentrations, it significantly inhibited putrescine incorporation (Table II) as was confirmed by an experiment performed for 3 h. Casein at 750 or 1,500 μg test tube^{-1} caused an inhibition of 33 and 37%, respectively, compared to untreated controls. It appeared not to be recognized as a substrate by the plant enzyme, as confirmed by the absence of radioactivity in the casein bands separated on SDS-PAGE after the assay performed with the 22,000*g* supernatant (Fig. 1).

Table I. *Putrescine-Binding Capacity as a Function of DTT Concentration*

Various DTT concentrations were tested both in the crude extract and in the assay mixture; 0.3 μM [^{3}H]putrescine and 200 μM cold putrescine were added in the test tube. The averages were significantly different (1% Student's *t*-test).

DTT Concentration		Bound [^{3}H]Putrescine	
In the crude extract	In the assay mixture		
mM		*dpm 30 min^{-1}*	*pmol mg protein^{-1} 30 min^{-1}*
10	13	5,605 ± 598	0.165
30	10	45,997 ± 5,809	1.026
30	40	3,457 ± 396	0.093

Table II. *Putrescine-Binding Capacity as a Function of N,N'-Dimethylcasein Concentration*

The assay was performed for 30 min; 0.2 μM [^{3}H]putrescine and 200 μM cold putrescine were added in the test tube. Different letters indicate averages significantly different from each other (5%, Student's *t*-test).

N,N'-dimethylcasein	Bound [^{3}H]Putrescine	
μg test tube^{-1}	*dpm 30 min^{-1}*	*pmol mg protein^{-1} 30 min^{-1}*
0	52,712 ± 9,874a	1.321
75	50,602 ± 5,828a	1.284
150	59,742 ± 12,222a	1.520
450	48,601 ± 4,687a	1.231
750	21,743 ± 11,755ab	0.536
1,500	15,089 ± 7,597b	0.363

The calcium requirement was checked, since animal tranglutaminases are Ca^{2+}-dependent. Some animal tranglutaminases do not require exogenously supplied Ca^{2+} (23, 25) and/or are inhibited by high Ca^{2+} concentrations (9, 14). The total endogenous Ca^{2+} content in the sprout apices was approximately 1 mM in the crude extract filtered through cheesecloth and was diluted to 300 μM in the assay mixture. Two hundred μM Ca^{2+} is usually sufficient to activate transglutaminases in animal systems (8, 17). In our system, the addition of up to 5 mM $CaCl_2$ either to the crude extract or after its elution from a Sephadex G25 column (Fig. 2 and inset) did not significantly affect activity.

Experiments were also performed with chelating agents. EDTA markedly inhibited the activity of the crude extract at 15 mM or more (Fig. 3) and EGTA, up to concentrations much higher (4 or 10 mM) than those of endogenous Ca^{2+} which should reduce the activity to zero, caused instead a very small but significant inhibition (respectively, 16 and 17%). These data suggest that Ca^{2+} is not an absolute requirement for this activity.

Controls and Time Dependence. The controls, performed in the absence of the plant extract, always gave a very low average value: 710 dpm (30 min)$^{-1}$. This value was not affected by the presence of different molarities of dimethylcasein, DTT, and putrescine, of $CaCl_2$ or its chelating agents, by different pH

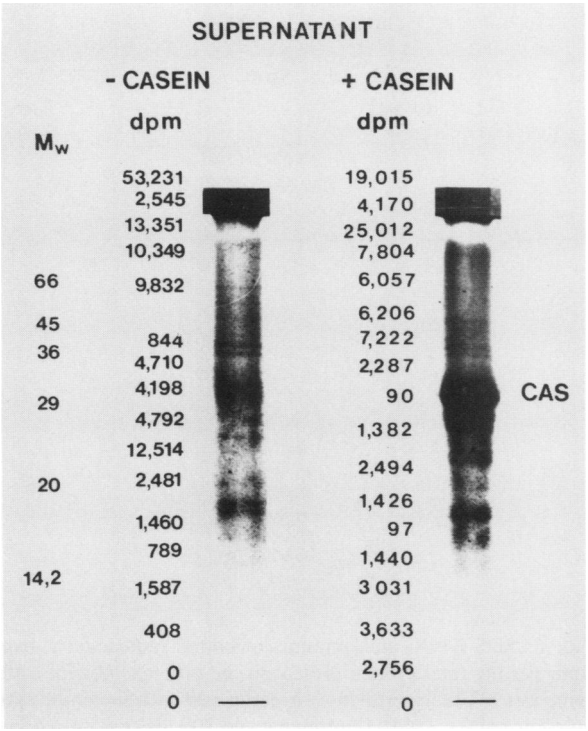

FIG. 1. SDS-PAGE pattern and amounts of bound radioactivity, expressed as dpm per mg total protein present in the products obtained after the enzyme assay. The experiment was performed with the supernatant in the presence of 0.6 μM [^{3}H]putrescine and 200 μM cold putrescine, with or without 750 μg *N,N'*-dimethylcasein (test tube)$^{-1}$.

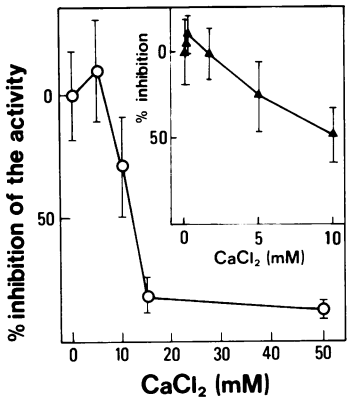

FIG. 2. Percentage inhibition of the putrescine-binding capacity of the crude extract as a function of $CaCl_2$ concentration added to the assay mixture. 0.4 μM [^{3}H]putrescine and 200 μM cold putrescine were added to each test tube. (O), Crude extract; (▲), crude extract eluted from a Sephadex G25 column.

values, nor by the addition of a previously boiled extract. It also remained constant at various intervals during the time course of the assay, as shown in Figure 4, where the time dependence of the activity in the crude extract is also reported.

The enzyme activity remained substantially unaltered when the crude extract was stored, while avoiding contact with air, at various temperatures (−80°C, −20°C, +4°C, +20°C) for 24 h.

Distribution of the Polyamine-Binding Activity. The polyamine-binding activity was mainly located in the 22,000*g* supernatant of the crude extract (65%). Animal transglutaminase activity is also mainly localized in the supernatant obtained from centrifugation at various speeds (from 12,000–105,000*g*) (5, 8,

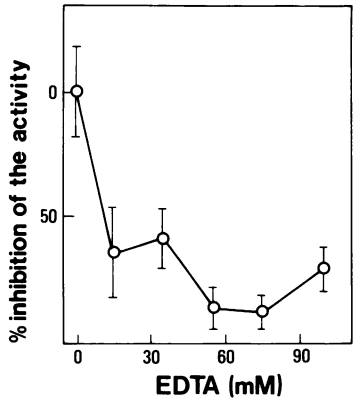

FIG. 3. Percentage inhibition of the putrescine-binding capacity of the crude extract as a function of the EDTA concentration in the assay mixture. 0.4 μM [^{3}H]putrescine and 200 μM cold putrescine were added to each test tube.

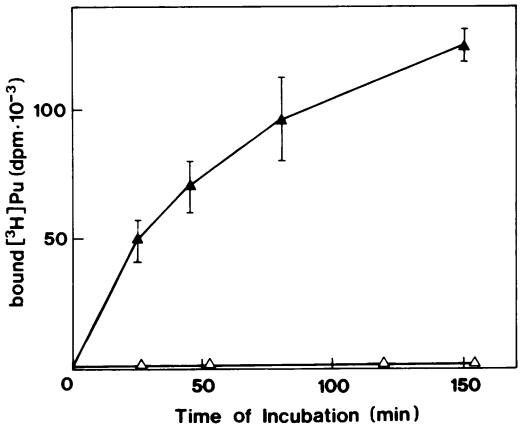

FIG. 4. Putrescine-binding capacity of the crude extract as a function of time. 0.4 μM [^{3}H]putrescine and 200 μM cold putrescine were added to each test tube. (▲), Crude extract; (△), control (performed without crude extract). Pu, putrescine.

15). In addition, Juprelle-Soret *et al.* (14) found a transglutaminase activity in rat liver crude homogenates and another in lysosomes. Boiling the crude extract, its 22,000*g* supernatant, or its precipitate for 10 to 20 min destroyed all capacity to induce the binding of putrescine, thus demonstrating the reaction was enzyme catalyzed. The addition of an equal volume of boiled supernatant to the crude extract reduced the binding activity by 30% (with respect to a control consisting of equal volumes of unboiled supernatant and extraction buffer). This can be due to the presence of competitive amines in the supernatant or to inhibitory oxidation products formed as a result of boiling.

Dependence of the Activity on the Amine Substrate Concentration. An analysis of the data shows that the activity was dependent on the putrescine concentration in the assay mixture (Fig. 5, inset). Plotting activity *versus* low putrescine concentrations yielded a sigmoidal curve (Fig. 5, unconnected square symbols). This suggests the enzyme is cooperative. nevertheless, the interpolation of the data based on a Hill plot gave a poor fit. In fact, at putrescine concentrations lower than 0.5 mM, the curve resembled more a hyperbola than a sigmoid. This could be explained by the presence of two enzymes, or two forms of one enzyme, contributing to the total activity, one with high affinity and low velocity hyperbolic kinetics (component A, evident at low concentrations) and the other with low affinity and high velocity sigmoidal kinetics (component B, evident at high concentrations).

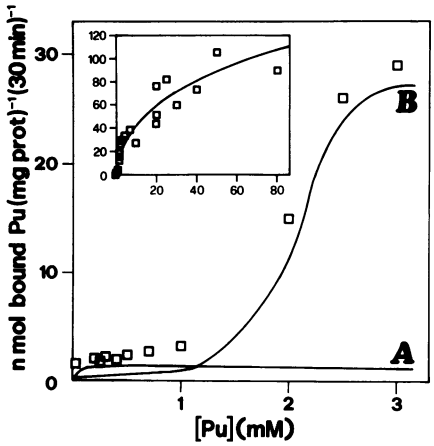

FIG. 5. Putrescine-binding capacity of the crude extract in relation to putrescine concentration (endogenous putrescine concentration in the crude extract was 7 μM: [^{3}H]putrescine concentration was 0.4 μM). (□), Experimental data. A and B, curves for components A and B, calculated as described in the text; inset, overall curve; Pu, putrescine.

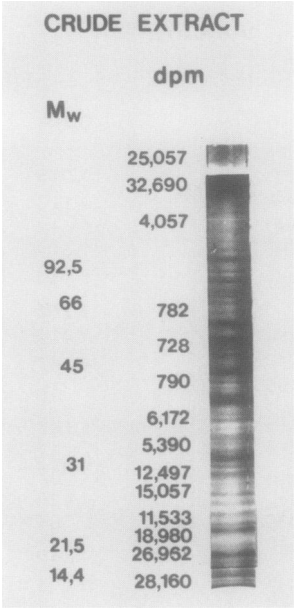

FIG. 6. SDS-PAGE and amounts of bound radioactivity, expressed as dpm per mg total protein present in the products obtained after the enzyme assay. The experiment was performed with the crude extract in the presence of 0.6 μM [^{3}H]putrescine and 200 μM cold putrescine.

As described in Segel (29), it was assumed that component B can be neglected at low substrate concentrations and a Lineweaver-Burk plot was used to obtain the apparent kinetic parameters of component A (K_m = 0.21 mM and V_{max} = 2.4 nmol mg protein^{-1} h^{-1}).

With these theoretical parameters, the curve for component A was constructed (Fig. 5, curve A). The values on this curve were then subtracted from the overall activity curve to give the curve for component B (Fig. 5, curve B) whose values were used to construct a Hill plot. Based on the latter, the following were calculated: $[S]_{0.5}$ = 22 mM and n = 2, confirming a positive cooperativity. The correlation coefficients for curves A and B were 0.99 and 0.95, respectively.

For animal transglutaminases, K_m values range from 0.09 mM (19) to 0.7 mM (14) frequently with *N,N'*-dimethylcasein as the protein substrate. Furthermore, the existence of multiple forms of the enzyme has also been reported in guinea pig prostate and sea urchin egg (17). In evaluating these data one must remember that in *Helianthus*, putrescine may be bound to more than one endogenous substrate and not to casein, as shown by SDS-PAGE (Figs. 1 and 6).

Specificity of the Activity for the Amine Substrate. The transglutaminases of animal origin are known to catalyze the binding of many amines other than polyamines (18). In extracts of *H. tuberosus* sprout apices, 5 mM histamine, a competitive inhibitor of transglutaminase, caused a 64% inhibition of the activity. Similarly, Paonessa *et al.* (23) observed in rat sperm a 79% inhibition of spermine conjugation at the same histamine molarity.

To verify the specificity toward various polyamines, the binding efficiency for equimolar concentrations of [^{14}C]-putrescine, -spermidine, or -spermine were compared. Spermine and spermidine were much more efficiently linked than putrescine. The relative binding activities were: putrescine 34, spermidine 100, and spermine 87, while in guinea pig liver transglutaminase, the corresponding values were 38, 100, and 92, respectively (23).

Analysis of the Complexes. Putrescine, or its derivatives, can also bind to proteins by ionic linkages as demonstrated by Balestreri *et al.* (2) in *Medicago sativa* leaves where protease activity was inhibited by binding of spermine to the enzyme. This inhibition, due to a spermine-induced conformational change of the protease molecule, was prevented by the ionic strength of a 50 mM Tris buffer.

To verify the occurrence of such ionic linkages in *H. tuberosus* extracts, the reaction product, previously precipitated with TCA, was washed with NaCl. There was no significant decrease in the amount of radioactive complexes recovered on the filters. However, if NaCl was added prior to filtering and TCA treatment, considerably fewer labeled complexes (22%) were recovered, but this was due to the fact that, in this way, some of the labeled proteins pass through the filters (50% of those present in the assay mixture compared to 1 to 5% in the controls not washed with NaCl).

In addition, when the reaction products (obtained from assays performed with mixtures having three different final pH values) were washed with cold putrescine at high concentrations (25-fold that of the assay mixture), no further release of labeled putrescine was observed (Table III).

Acid hydrolysis, which destroys the linkage of polyamines to

Table III. *Putrescine-Binding Capacity as a Function of Cold Putrescine Washing*

The assays were performed at three different pH values and the resulting products, collected on the filters, were washed, as indicated, with 100 volumes 5 mM cold putrescine; 0.3 μM [^{3}H]putrescine and 200 μM cold putrescine were added in the test tube. At each pH value, the average of incorporation in the samples washed with or without putrescine was never significantly different one from the other (5%, Student's *t*-test).

pH of the Assay Mixture	Bound [^{3}H]Putrescine	
	dpm 30 min^{-1}	*pmol mg protein*$^{-1}$ *30 min*$^{-1}$
6.6		
With Pu	1,387 ± 405	0.007
Without Pu	1,411 ± 151	0.007
7.8		
With Pu	61,713 ± 9,937	0.845
Without Pu	59,180 ± 16,559	0.810
8.0		
With Pu	34,516 ± 2,264	0.356
Without Pu	27,768 ± 5,660	0.263

partner molecules in the labeled complexes, and the subsequent comigration of labeled compounds with cold standard polyamines on mono- and bi-dimensional TLC plates allowed us to determine that most of the label in the benzene phase was still putrescine, whereas spermidine and spermine together represented only 1 to 2%; the remaining label was probably due to putrescine derivatives formed during the assay procedure.

The electrophoretic pattern of the reaction products obtained using either the crude extract or its supernatant exhibited two labeled regions (Figs. 1 and 6). In the first, there were endogenous substrates that were either too heavy or too insoluble to enter the stacking gel and others which entered the running gel and had a mol wt higher than 92.5 kD. Similarly, Folk (11) found that 40% of the label, after transglutaminase-mediated binding, associated with proteins that could not enter the gel. Birckbichler *et al.* (5) suggested that rat liver transglutaminase itself, which has a high mol wt, may also act as the acceptor substrate. In the second region, labeled bands corresponding to molecules having a mol wt of approximately 30 kD or less were observed. As expected, free putrescine was not present in the gel. Finally, SDS-PAGE was carried out on a mixture of labeled putrescine previously incubated with standard reference proteins used as mol wt markers. Neither labeled bands nor free putrescine were detected, demonstrating that putrescine binding is dependent on the enzyme activity present in the plant extract.

All these data indicate that, in *H. tuberosus*, the capacity to covalently link polyamines to endogenous substrates exists and that it has an efficiency similar to that of a mouse liver crude extract (that we preliminarily measured). This plant enzyme activity shows some similarities with mammalian transglutaminases, even though the fact that it does not recognize *N,N'*-dimethylcasein as a substrate and that it does not require exogenous Ca^{2+} may indicate that this activity is different from that of animal transglutaminases which, incidentally, are the only known enzymes able to catalyze the covalent binding of polyamines. The requirement for exogenous Ca^{2+}, however, may not be a discriminating factor since some unpurified animal transglutaminases are independent of added Ca^{2+} (23, 25). In the final analysis, only the identification of glutamyl-polyamine derivatives will prove beyond any doubt the existence in plants of a transglutaminase activity.

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