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Communication

Evidence for Transglutaminase Activity in Plant Tissue

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ISAAC ICEKSON AND AKIVA APELBAUM*
Department of Fruit and Vegetable Storage, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

ABSTRACT

An extract prepared from the apical meristematic region of etiolated pea seedlings was able to catalyze the incorporation of putrescine into trichloroacetic acid precipitable material. The enzyme was found to be soluble and followed a typical Michaelis-Menten kinetics when *N-N*-dimethyl casein was used as a substrate. Its activity was promoted by Ca^{2+} and inhibited by Cu^{2+} and DL-dithiothreitol. Other polyamines competed with putrescine as substrates and cadaverine was the most potent inhibitor of putrescine incorporation. Plant transglutaminase is capable of recognizing specific sites in substrates described for animal transglutaminase, like insulin, fibrinogen, pepsin, and thrombin. However, it can also use as substrates cellulase and creatine kinase which have not been described for transglutaminase from other sources.

Transglutaminase (TGase 2.3.2.13) is an enzyme found intracellularly and extracellularly in various organisms and catalyzes the covalent attachment to proteins and polypeptides of a series of substances containing primary amine groups. This enzyme promotes the formation of amide linkages, generally in a Ca^{2+}-dependent fashion, between the primary amine of an amine donor substrate and the γ-carboxamide group of peptide-bound endo-glutamine residues in proteins or polypeptides that are the amine acceptors (5, 10). Polyamines have been shown to serve as physiological substrates of transglutaminase (6, 15) and the incorporation of radioactively labeled polyamines is commonly used as a detection method for the activity of transglutaminases (11, 12). Polyamines appear to play an essential role in growth and cell division process in animals, microorganisms, and plants (2, 7). It was suggested that the polyamines may exert their regulatory action by a transglutaminase-mediated process of post-translational modification (addition of polyamine moieties) of enzymes and structural proteins (13, 14). Despite the presence of transglutaminases in an extremely broad spectrum of living organisms (5, 10, 15, 18) no reports as to the existence of transglutaminases in plants have been published. As we have studied in the past the physiology and biosynthesis of polyamines in plants under hormonal stimuli (1) and in view of the above, a study was undertaken to pursue the possible involvement of transglutaminases in the effects of polyamines on plant growth and development.

We present here, for the first time, evidence indicating that an enzyme with characteristics similar to those found in most transglutaminases described so far is found in plant meristematic tissues.

MATERIALS AND METHODS

Seeds of *Pisum sativum* (var 'Kelvedon Wonder') were soaked for 6 h in tap water, planted in pots containing moist vermiculite, and grown in the darkness at 23°C and 80% RH. The apical meristematic hook regions from 7-d-old etiolated pea seedlings were excised and homogenized in a chilled mortar and pestle with 4 to 5 volumes of Tris 0.1 M (pH 8.5). The homogenate was centrifuged at 2000 rpm at 4°C for 10 min and the supernatant was assayed for transglutaminase activity. Transglutaminase activity was assayed by following the rate of incorporation of ^{3}[H] putrescine into TCA precipitable protein. The assay was conducted following the modified filter paper method described by Lorand *et al.* (11). The assay contained, in a total volume of 150 μl, 45 mM putrescine containing 2 μCi of [^{3}H]putrescine (18.6 μCi/mmole), up to 600 μg of plant protein in 75 μl homogenization buffer, up to 0.95 mg *N-N*-dimethylated casein, 1 μmole $CaCl_2$, and 62.5 μmol Caps[1] (pH 8.5). Extracts were incubated at 35°C for 30 min, then 60-μl aliquots were spotted on Whatman No. 1 paper presoaked in 10% TCA and immediately immersed in ice-cold TCA (10%). The filters were washed twice in 5% TCA and once in an ethanol/acetone 1:1 mixture, and once in acetone. The filters were then dried, placed in scintillation vials containing 3 ml Aqualuma (Lumac B.V., Holland), and counted in a Kontron scintillation counter. The transglutaminase activity was expressed as nmol of ^{3}[H]putrescine incorporated per mg of plant protein per h. The activity was linear up to 45 min at 35°C in the range of protein content between 5 and 600 μg per assay. Blank values (up to 200 dpm) of acid-treated or boiled enzymes preparation were always subtracted. The data presented are from single experiments, which are representative of a group of three experiments, each involving triplicate sets of experimental treatments and controls.

RESULTS AND DISCUSSION

Extracts prepared from the apical meristematic tissue of etiolated pea seedlings were able to catalyze the incorporation of ^{3}[H]putrescine into TCA precipitable material in a time and protein concentration-dependent manner. The addition of *N-N*-dimethyl casein, commonly used as a substrate for transglutaminases from various sources (18), greatly enhanced putrescine incorporation (Table IA). When the incorporation of [^{3}H]putrescine into *N-N*-dimethyl casein by pea seedlings transglutaminase was studied as a function of putrescine concentration, it was found to follow the typical Michaelis-Menten kinetics (not shown). The values obtained from a Lineweaver-Burk plot of the data gave an apparent V_{max} of 41 nmol/mg protein h and an apparent K_m of 9.63 mM putrescine.

Transglutaminase is generally described as being Ca^{2+} ion-

[1] Abbreviation: Caps, 3-cyclohexylamino propanesulfonic acid.

Table I. *Influence of Various Effectors on Plant Transglutaminase*

The complete system contained in 150 μl: 45mM putrescine, containing 2 μCi of ^{3}[H]putrescine, 400 μg/ml plant protein, 6.3 mg/ml *N-N*-dimethyl casein, 6.6 mM $CaCl_2$, and 0.4 M Caps (pH 8.5). The 100% = 58 nmol putrescine/mg protein·h.

System	Percent of Control
A	
Complete system	100
− *N-N*-dimethyl casein (0 mg/ml)	27
+ *N-N*-dimethyl casein (4 mg/ml)	75
B	
− Ca^{2+}	53
− Ca^{2+} + EDTA 10 mM	82
− Ca^{2+} + EGTA 8 mM	84
+ Ca^{2+} 1.66 mM	78
+ EDTA 10 mM + Ca^{2+} 10 mM	98
+ EGTA 4 mM + Ca^{2+} 10 mM	98
+ *o*-phenanthroline 1 mM	78
+ *o*-phenanthroline 4 mM	36
+ *o*-phenanthroline 8 mM	27
+ *o*-phenanthroline 4 mM + Ca^{2+} 10 mM	16
C	
+ DTT 1.5 mM	58
+ DTT 16.5 mM	60

Table II. *Effect of Polyamines on Plant Transglutaminase Activity*

The complete system contained in 150 μl: 45 mM putrescine, containing 2 μCi of ^{3}[H]putrescine, 400 μg/ml plant protein, 6.3 mg/ml *N-N*-dimethyl casein, 6.6 mM $CaCl_2$, and 0.4 M Caps (pH 8.5). The 100% = 51 nmol putrescine/mg protein h.

Amine	Concentration	Enzyme Activity
	mM	*% control*
	10	93
	20	94
Diaminopropane	100	59
	10	74
	20	61
Cadaverine	100	37
	10	90
	20	89
Spermidine	100	74
	10	77
	20	72
Spermine	100	68

dependent enzyme; however, some cases of calcium-independent activity have been described (4, 18). The transglutaminase activity displayed by pea seedlings extract shows stimulation by Ca^{2+} when measured with *N-N*-dimethyl casein as substrate (Table IB), but was not increased even in the presence of 17 mM Ca^{2+}, when studied as incorporation of ^{3}[H]putrescine into the proteins present in the plant extract (not shown). Chelators, like EDTA and EGTA, produced very little inhibition of putrescine incorporation into *N-N*-dimethyl casein and addition of calcium ions caused complete reversal of this effect (Table IB). *o*-Phenanthroline caused a more pronounced inhibition which could not be reversed by simultaneous addition of Ca^{2+} ions (10 mM). An inhibitory action of low levels of Cu^{2+} ions has been described in guinea pig liver transglutaminase (5). In pea seedlings transglutaminase, the efficient Cu^{2+} ion chelator, diethyldithiocarbamate, produced a 70% stimulatory effect at low concentrations

Table III. *Plant Transglutaminase Activity in the Presence of Different Substrates*

The complete system contained in 150 μl: 45 mM putrescine, containing 2 μCi of ^{3}[H]putrescine, 400 μg/ml plant protein, 6.3 mg/ml *N-N*-dimethylated casein, 6.6 mM $CaCl_2$, and 0.4 M Caps (pH 8.5). The 100% value = 68 nmol putrescine/mg protein·h.

Substrate	Percent Activity
N-N-Dimethyl casein	100
Fibrinogen	67.7
Thrombin	90.0
Pepsin	110.8
Insulin	149.5
Creatine kinase	86.7
BSA	0
Catalase	0
Cellulase	163.3

(0.45 mM) (not shown), indicating that plant transglutaminase may be inhibited by trace amounts of Cu^{2+} ions. An attempt was made in this study to stabilize the plant transglutaminase activity by adding DTT. However, addition of 1.5 mM DTT produced approximate 40% inhibition of transglutaminase activity (Table IC). This suggests that transglutaminase from pea seedlings may be somewhat different from that of guinea pig (5).

It appears that plant transglutaminase uses polyamines as amine donors since ^{3}[H]putrescine incorporation is retarded in the presence of other di- or polyamines. Of the diamines tested (Table II) cadaverine showed a higher apparent inhibition of transglutaminase than diaminopropene. The two polyamines tested showed a smaller inhibition of putrescine incorporation than cadaverine. When pea seedlings transglutaminase was assayed in the presence of 13 different amino acids (25 mM) to test the capacity of amino acids to serve as amine donors, only cysteine produced an 85% inhibition; none of the other amino acids tested had any significant effect (not shown). It is of interest to compare this observation to the report of a cysteine residue at the active site of guinea pig transglutaminase (5) and to speculate whether there is an essential Zn^{2+} ion in the vicinity of the active site of plant transglutaminase that could be bound by *o*-phenanthroline (Table I) or by cysteine.

A further similarity between plant transglutaminase activity and mammalian transglutaminases was apparent when various proteins were used as substrates for plant transglutaminase. It appears (Table III) that plant transglutaminase is capable of recognizing specific sites in *N-N*-dimethyl casein, as well as sites in other proteins that have been described as substrates for animal transglutaminases, like insulin, pepsin (3), fibrinogen and thrombin (10). In addition, plant transglutaminase can use as substrate other proteins not yet described for transglutaminase from animal origin, like cellulase and creatine kinase (Table III). Native BSA and catalase did not serve as substrate for plant transglutaminase. The fact that creatinine kinase can serve as a substrate for transglutaminase may indicate a possible mechanism of regulation on a key metabolic enzyme that has been shown to be an early marker for hormone stimulation of cell metabolism (9). The possibility that creatine kinase activity could be modulated by covalent addition of diamine or polyamine residues should certainly be explored experimentally.

Transglutaminase has been described mostly as a soluble enzyme in animals (5) or in several organisms as being at least partially bound to membranes (4, 16), either in the lysosomes or in the mitochondria (8). It was therefore of interest to study the intercellular localization of transglutaminase in plants. When the enzyme was extracted under conditions that preserve the integrity of pea seedlings mitochondria (17), over 90% of the activity was

found to be soluble in the supernatant and only 3% of the activity remained associated with the 27,000*g* pellet (not shown). Therefore, it appears that the plant transglutaminase activity is soluble, similar to that described for most animal transglutaminases.

The evidence we present in this communication strongly suggests the existence of transglutaminase activity in plant seedlings.

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