Pea Seedling Extracts Catalyze Protein Amine Binding and Protein Cross-Linking. 1. Evidence for the Role of a Diamine Oxidase

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An enzymatic fraction (PPE) obtained by selective precipitation from a pea seedling extract was shown to catalyze putrescine incorporation. In order to elucidate the type of enzyme, transglutaminases or diamine oxidases (DAO), involved in this activity, the ability of this PPE fraction to catalyze their characteristic reactions, amine binding, deamidation, protein cross-linking, and oxidation was investigated. The use of specific enzyme inhibitors pointed out that only DAO was present in the extract and should be involved in the protein cross-linking reactions. This reaction was only catalyzed in the presence of diamines, especially putrescine and cadaverine, or polyamine like spermidine.

Keywords: *Guinea pig liver transglutaminase; pea seedling diamine oxidase; amine binding; cross-linking; deamidation; Pisum sativum*

INTRODUCTION

The enzymatic incorporation of amines into proteins is catalyzed *in vivo* by transglutaminases (TGases) but can also occur as a secondary reaction following the oxidation of diamines into aldehydes by the diamine oxidases (DAO). TGases catalyze, generally in a Ca^{2+} dependent manner, an acyl-transfer reaction in which the γ-carboxamide groups of peptide-bound glutaminyl residues are the acyl donors and small molecular weight amines are the acyl acceptors. Besides their amine-binding ability, TGases are also able to cross-link proteins through ϵ-(γ-glutamyl)lysyl bonds and to deamidate glutaminyl residues (Kurth and Rogers, 1984). DAO catalyzed the oxidation of diamines into an amino-aldehyde intermediate, with release of NH_3 and H_2O_2 (Hill, 1971). These aldehydes could be further oxidized or react spontaneously with protein amino groups giving rise to a Schiff base formation (Williams-Ashman and Canellakis, 1980).

In plants, enzymatic activity of amine binding into proteins was found in many types of tissues. Cohen *et al.* (1982) first detected a radioactive polyamine incorporation into the acid-precipitated fraction of *Brassica pekinensis* chloroplast extract. This reaction was time and light dependent. The authors did not consider it as a TGase-like reaction because it was not calcium dependent. A similar activity was found in the *Helianthus tuberosus* tubers (Serafini-Fracassini and Mosseti, 1985). In 1987, the binding of amines to proteins was observed in etiolated pea (*Pisum sativum*) apical meristems by Icekson and Apelbaum (1987) and was characterized as a Michaelis−Menten kinetic type enzyme, calcium independent. In recent studies, this activity was identified in tissues of *H. tuberosus* L. (Serafini-Fracassini *et al.*, 1988), tobacco (*Nicotiana tabacum*) (Apelbaum *et al.*, 1988), alfalfa (*Medicago sativa* L.) (Margosiak *et al.*, 1990), spinach (*Spinacia oleracea*), broccoli (*Brassica oleracea*), silver beet (*Beta vulgaris* L.) (Signorini *et al.*, 1991), lupine (*Lupinus albus*) (Pallavicini and Trentin, 1990), and *Chrysanthemun* (Aribaud *et al.*, 1995). In all cases, this activity was referred to as TGase-like activity, although it was not strictly dependent on Ca^{2+} ions like animal enzymes.

Most of the studies on DAO concern physiological and pathological processes in animals and plants. Recently, a DAO of lupine seedling (*L. albus*) was reported to catalyze incorporation of [^{14}C]putrescine into dimethylcasein (Siepaïo and Meunier, 1995); the resulting protein−aldehyde conjugate has not yet been identified. By using such lupine enzymatic extracts, some authors also observed protein cross-linking and claimed that it results from TGase activity, although the glutamyl−lysine conjugate has not been isolated (Pallavicini *et al.*, 1990; Siepaïo and Meunier, 1995).

As seen from these previous studies, the type of enzyme responsible for amine binding in plant extracts is controversial, Tgase or DAO. The presence of polyamine oxidases (PAO), able to oxidize the two primary amino groups of a diamine, is excluded because this enzyme does not occur in *Leguminosae*, their presence being limited to cereals (Smith, 1985). Using a pea seedling extract, the aim of this study was to discriminate the respective role of TGase and DAO in amine binding. The activities of cross-linking and deamidation were also investigated and compared with animal TGase.

MATERIALS AND METHODS

Protein Determination. Proteins in the crude extract were measured using the Bradford method (Bradford, 1976). Bovine serum albumin was employed as reference.

Preparation of Substrate Proteins. Native casein was purchased from Sigma; 11S soy protein fraction was prepared according to Kim and Kinsella (1986) and total gliadins according to Popineau and Pineau (1985). Native proteins (10 mg/mL) were acylated at 28 °C with acetic or citraconic

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S0021-8561(96)00201-4 CCC: $12.00 © 1996 American Chemical Society

anhydride, as described by Brinegar and Kinsella (1980), in 50 mM Tris-HCl, pH 8.5. The pH was controlled and maintained in the range of 7.5−8.2 by addition of 2 N NaOH. The excess of reagent was eliminated by dialysis against water for 48 h, and the modified proteins were lyophilized. The acylation degree was determined by the TNBS method (Barber and Warthesen, 1982). All samples were acylated at least at 90%.

Plant Material. The crude extracts (CE) were obtained as described by Icekson and Apelbaum (1987), with slight modifications (Chiarello, 1994). Seeds of *P. sativum* (var. Arkel) were soaked overnight in running water, cleaned with sodium hypochlorite (10%) for 15 min, placed on trays covered with moist cotton layers, and grown in darkness at 22 °C and 80% RH. After 10 days, the apical meristematic hook regions were excised and frozen at −80 °C until utilization. The frozen etiolated pea meristems were homogenized in a chilled mortar with 1 vol of extraction buffer—50 mM Tris-HCl (pH 8.5)—containing antiproteases (8 mM disodium EDTA, 6 μM leupeptin, and 6 μM pepstatin A). The homogenate was centrifuged at 12000*g* for 20 min. The supernatant was recovered as CE. Some of the nonenzymatic proteins were further eliminated by an isoelectric precipitation procedure. Initially, chilled 0.5 N HCl was added drop by drop to the CE, until reaching pH 4. The resulting suspension was gently stirred for 30 min, and insoluble proteins were eliminated by centrifugation at 12000*g* for 20 min. Subsequently, the pH was dropped to 3 by addition of 0.5 N HCl, and after 30 min, the precipitate was eliminated by centrifugation under the same conditions as above. All of these steps were carried out at 4 °C. This two-step precipitation procedure was preferred to a single-step one because of a higher activity recovery yield. The supernatant, containing the active enzyme fraction, called partially purified extract (PPE), was adjusted to pH 8 with chilled 2 N NaOH and then frozen to −80 °C until utilization.

Guinea Pig Liver Transglutaminase (GPL). GPL was purified from fresh guinea pig livers according to the method of Larré *et al.* (1992). The activity was determined according to Larré *et al.* (1992) and expressed in nkat; 1 kat is defined as the amount of enzyme that binds 1 mol of putrescine/mg of acetylated casein/s under experimental conditions.

Amine-Binding Reactions. The enzyme activity was measured by mixing 75 μL of CE or PPE, 1 mg of citraconylated casein, 40 mM of cold amine, and 200 nCi of ^{14}C-radiolabeled amine in 100 mM citrate buffer (pH 6.5) in a total volume of 150 μL. When GPL TGase was analyzed, 6.7 mM $CaCl_2$ and 1 mM DTT were added to the reaction. Controls were carried out without casein to quantify the autoincorporation of [^{14}C]amine into the plant proteins. Reaction mixtures were incubated at 37 °C for 1 h. Aliquots of 50 μL of the mixture were put onto filter paper disks which were treated as described by Larré *et al.* (1992). All assays were made in duplicate. The experimental data were corrected for nonspecific adsorption of the radiolabeled probe on the substrate. Under the chosen incubation conditions, the reaction of the pea enzyme proceeded at a linear rate up to 60 min. Various amines were tested, *n*-butylamine (8 mCi/mmol; Sigma), lysine (319 mCi/mmol; Amersham), cadaverine (108 mCi/mmol; Dositek), spermidine (110 mCi/mmol; Amersham), and spermine (90 mCi/mmol; Dositek). The proteins employed were casein, total gliadins, and 11S soy protein fraction. For kinetic assays, aliquots of the reaction mixtures were taken at 0, 15, 30, 60, 120, and 240 min and analyzed. Amine-binding ability was determined by the filter paper method, as described above.

SDS−Polyacrylamide Gel Electrophoresis. SDS−PAGE was carried out in homogeneous polyacrylamide (12−15%) slab gels with 5% stacking gels according to Laemmli (1970), after reduction of the samples with 7.5% β-mercaptoethanol. The gels were fixed in trichloroacetic acid (12.5%, w/w) during 30 min and stained overnight with 0.3% Coomassie blue G250 in 50% methanol. The destaining was performed in a water/ethanol/acetic acid mixture (4.5:4:5, v/v/v).

Determination of ϵ-(γ-Glutamyl)lysyl Bond Measurement. To estimate the number of ϵ-(γ-glutamyl)lysyl bonds formed by the various enzymatic preparations, purified β-casein

Table 1. Amine-Binding Activity of Crude and Partially Purified Pea Seedling Plant Extracts

plant material	amine-binding specific activity (pkat/mg)[a]	residual activity yield (%)
CE	21	100
CE + SEC[b]	80	13
CE + SEC + desalting[c]	8	1
PPE	228	90
PPE + UF 30 kDa[d]	72	36
PPE + UF 100 kDa[e]	91	28
PPE + dialysis[f]		
3.5 kDa	77	31
8−12 kDa	84	22

[a] Amine-binding specific activity is expressed in pkat/mg of substrate (casein and endogenous proteins)/mg of proteins in the enzymatic preparation. Reaction conditions: 75 μL of plant extract incubated at 37 °C for 1 h with 1 mg of citraconylated casein, 40 mM cold putrescine, 200 nCi of [^{14}C]putrescine, and 100 mM citrate buffer, pH 6.5, in a final volume of 150 μL. [b] Size exclusion chromatography: active fraction recovered after Superose 12 HR 10/30 elution with 100 mM Tris-HCl, 200 mM NaCl, pH 8.5. [c] Gel filtration column (PD 10, Pharmacia). [d] UF: ultrafiltration through 30 kDa molecular weight cutoff membrane. [e] UF: ultrafiltration through 100 kDa molecular weight cutoff membrane. [f] Dialyzed against 20 vol of 50 mM Tris-HCl, pH 7.5, containing antiproteases (8 mM EDTA, 6 μM leupeptin, 6 μM pepstatin A), during 72 h at 4 °C.

(Sigma) was employed as model substrate. The reaction products obtained after 240 min of incubation with GPL or PPE were dialyzed against water and lyophilized before analysis. They were submitted to a sequential proteolytic attack (subtilisin, pronase, leucine aminopeptidase, prolidase, and carboxypeptidase) according to the method of Griffin *et al.* (1982). Freeze-dried protein digests were derivatized by PITC (Bidlingmeyer *et al.*, 1987). Amino acids and ϵ-(γ-glutamyl)lysine dipeptide were separated and quantified by RP-HPLC on a C_{18} column (LiChroCART 250-4 RP-18, Merck) (0.4 × 25 cm). Elution was achieved by a gradient from buffer A [70 mM sodium acetate buffer (pH 6.4), 0.25 mL/L triethylamine] to buffer B (60% acetonitrile in water) at 44 °C. Standards of amino acids as well as of ϵ-(γ-glutamyl)lysine (Sigma) were used for the identification of the peaks and their quantitative evaluation.

Determination of Ammonia. The amount of ammonia released from the reaction mixture was determined enzymatically using glutamate dehydrogenase (EC 1.4.1.2) according to the method of Kun and Kearney (1974).

RESULTS

Enzymatic Extracts Preparation. In the preparation of crude extract, 75% of the amine-binding activity was detected in the supernatant. Assays of solubilization of the pellet were done to recover the remaining nonextracted activity by addition of detergents (Triton X 100, Tween 20, Brij 35, cholate) to the extraction buffer. The activity recovered was always lower than that obtained by washing the pellet with the initial extraction buffer (50 mM Tris-HCl, pH 8.5). The supernatant of the pea seedling crude extract was submitted to purification methods in order to isolate the enzyme responsible for amine-binding activity. Isoelectric precipitation of crude extract proteins at pH 3 led to a 10 times increase of the specific amine-binding activity in the supernatant designated as PPE. Further procedures based on molecular weight separation (size exclusion chromatography (SEC), ultrafiltration (UF)) resulted in a decrease of both specific activity and activity yield (Table 1). After ammonium sulfate precipitation (from 20 to 80% saturation), the activity recovered after dialysis was neither in the supernatant nor in the precipitated fractions. According to these

Table 2. Amine-Binding and Deamidation Reactions Catalyzed by GPL TGase and PPE Extract

substrate protein	bound amine level[a] (nmol of Put/ mg of substrate)		deamidation level[b] (nmol of NH_3/ mg of substrate)	
	GPL	PPE	GPL	PPE
native casein	152	104	163	0
acetylated casein	158	130	174	0
native soy 11S	55	75	60	0
acetylated soy 11S	204	102	225	0
native gliadins	170	23	178	0
acetylated gliadins	235	28	244	0

[a] The level of bound amine is expressed as putrescine nmol bound/mg of protein substrate after 4 h of reaction. Reaction conditions: 75 μL of PPE (*ca.* 15 pkat) incubated at 37 °C with 1 mg of added protein, 40 mM cold putrescine, 200 nCi of [^{14}C]putrescine, and 100 mM sodium citrate buffer (pH 6.5). For GPL TGase reaction, 30 μL of the enzyme preparation (*ca.* 100 pkat) was incubated at 37 °C with 1 mg of added protein, 40 mM cold putrescine, 200 nCi of [^{14}C]putrescine, 6.7 mM $CaCl_2$, 1 mM DTT, and 100 mM Tris-HCl buffer, pH 7. [b] The level of deamidation is expressed as the number of NH_3 nmol released/mg of protein substrate after 4 h of reaction. Reaction conditions: the same as stated above but without putrescine addition.

experimental difficulties, the further steps of this work were carried out with PPE as the enzymatic preparation.

Characterization of the Activities Catalyzed by the Pea Seedling Extract (PPE). Three types of reactions were investigated, amine binding, ammonium release, and cross-linking, using native or acetylated proteins as single substrates or with putrescine as second substrate.

Two-Substrate Conditions: (1) Amine Binding. Native and acetylated casein appeared to be good substrates for both PPE and guinea pig liver (GPL) TGase even if their reactivity was lower for the plant enzymatic extract (Table 2). On soybean 11S globulin, the activities observed were also quite similar but rather low for both enzyme preparations. By acylating this protein, the amine-binding level drastically increased for GPL (about 4 times) as previously observed by Larré *et al.* (1993a) on pea legumin. According to these authors, acylation by inducing the opening of the globular structure led to a higher reactivity of the oligomeric protein toward TGase. In contrast, the action of PPE appears to be less sensitive (only 40% increase) to this structure modification. A great difference between GPL enzyme and PPE was observed when nonglobular proteins like total wheat gliadin were used (Table 2). These proteins, very rich in glutaminyl residues, are good substrate for TGase (Alexandre *et al.*, 1993; Larré *et al.*, 1993b). In contrast, gliadin showed a rather poor reactivity with plant enzyme.

(2) Ammonia Release. The ammonia release was also followed during the binding reaction of putrescine and its value compared to the quantity of amine incorporated. In the case of GPL TGase, the total ammonia liberated after 4 h reaction reached 153 nmol/mg native casein, 90% of which was accounted for by the putrescine incorporated. Using glycinin as substrate, 62 nmol/mg was liberated and 64% of these corresponded to putrescine binding. With PPE, the total ammonia release was considerably higher and reached 1240 and 1125 nmol/mg for casein and glycinin, respectively, and no more than 10% of it can be accounted for by the amine incorporation (Figure 1). Using acylated proteins, the quantity of ammonia released is increased for GPL Tgase but not modified for PPE.

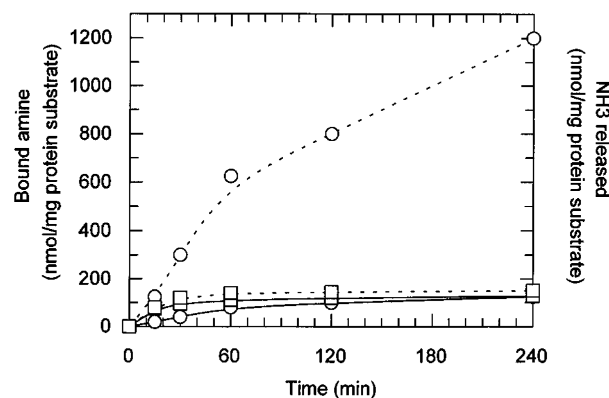

Figure 1. Kinetics of putrescine-binding (—) and NH_3 release (– – –) reactions catalyzed by PPE (○) or GPL TGase (□) toward native casein. Reaction conditions are the same as listed in Table 2.

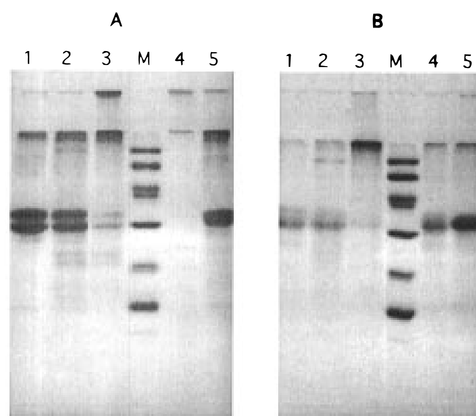

Figure 2. Native casein (A) and acetylated casein (B) cross-linking catalyzed by GPL TGase and PPE extract, with or without added putrescine. Four hour reaction products were submitted to SDS−PAGE and 20 μg of native casein loaded (A) or 10 μg of acetylated casein loaded (B): lane 1, control reaction, without enzyme addition; lane 2, PPE-catalyzed reaction; lane 3, PPE-catalyzed reaction with added putrescine; lane 4, GPL TGase-catalyzed reaction; lane 5, GPL TGase-catalyzed reaction with added putrescine; lane M, molecular weight protein markers (94, 67, 43, 30, 20, and 14.4 kDa, from the top). Reaction conditions are the same as listed in Table 2 but with putrescine addition when indicated.

(3) Protein Polymerization. The third activity interesting to compare between GPL TGase and PPE is their ability to cross-link proteins. The reaction performed on casein was followed by SDS−PAGE. By using this technique, polymer formation is characterized by the disappearance of the original casein bands and by the concomitant appearance of high molecular weight components at the top of the gel. The reaction products of the GPL TGase-catalyzed reaction were polymerized when amine was added; however, polymerization was limited because of the competition between the two acyl acceptors, diamine and the lysyl residues of the protein for the acyl-transfer reaction (Figure 2A, lane 5). With acetylated casein (Figure 2B, lanes 4 and 5), almost no polymerization was observed confirming the role of the lysyl residues in the formation of cross-links. When PPE was employed, polymerization was obtained in the presence of putrescine (Figure 2A, lane 3). Surprisingly, a high polymerisation level was also observed with acetylated casein (Figure 2B, lane 3), meaning that lysyl residues are probably not specifically involved in the cross-linking reaction.

By adding different types of amines (Figure 3), it clearly appeared that the diamines (putrescine and

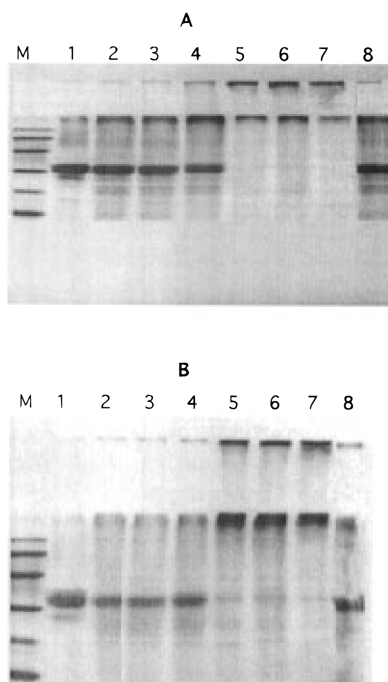

Figure 3. Native casein (A) and acetylated casein (B) cross-linking catalyzed by PPE extract, with various added amines. Four hour reaction products were submitted to SDS–PAGE (20 μg of protein loaded): lane 1, control reaction (with putrescine), without enzyme addition; lane 2, casein with PPE; lane 3, *n*-butylamine; lane 4, L-lysine; lane 5, putrescine; lane 6, cadaverine; lane 7, spermidine; lane 8, spermine; Lane M, molecular weight protein markers (94, 67, 43, 30, 20, and 14.4 kDa, from the top). Reaction conditions are the same as listed in Table 2 but varying added amine as indicated.

cadaverine) and the polyamine spermidine were more efficient in polymerizing casein, even when acetylated (Figure 3B, lanes 5–7). When lysine was employed, a very low polymerization was observed when native casein (Figure 3A, lane 4) was used as substrate. In the same way, the monoamine *n*-butylamine (lane 3) and the spermine (lane 8) induced a very weak and limited cross-linking reaction.

Single Substrate Conditions: (1) Ammonia Release. In order to compare the deamidation potentialities of PPE extract and GPL TGase, their activities were tested in the absence of added primary amine molecules. In the case of GPL TGase, the amount of ammonia released is of the same order of magnitude as the amount of amine previously bound to the protein (Table 2), indicating that the glutaminyl reaction sites were rather similar in both reactions. For pea enzymatic extract, no ammonia was produced when proteins were used as single substrates (Table 2).

(2) Protein Polymerization. Without any addition of external amine in the reaction mixture, GPL TGase polymerized almost completely native casein (Figure 2A, lane 4), but no cross-linking was detected when PPE was employed (Figure 2A, lane 2, and Figure 2B, lane 2).

The presence of glutamyl–lysyl conjugates in the products of the enzymatic reactions was checked, taking GPL-treated casein as reference. By analysis of the proteolytic hydrolysate from β-casein cross-linked by GPL TGase, the presence of γ-glutamyl-lysine was detected at a level of 49 nmol/mg β-casein, after 4 h reaction. When PPE-treated β-casein was analyzed, no trace of γ-glutamyl-lysine could be identified.

Effect of Specific Inhibitors and Activators of TGases and DAO on the PPE Activities. According to the previous results, it clearly appeared that the pea enzymatic extract does not catalyze the three types of reactions tested in the same way that animal TGase does. In order to better characterize the type of enzymatic activities involved in these reactions, the amine-binding capacity of the PPE was studied in the presence of specific inhibitors or activators of TGase and DAO (Table 3).

Table 3. Effect of Inhibitors and Stabilizing Agents on PPE and GPL TGase Amine-Binding Reactions

			residual amine-binding level[a] (%)	
chemical added	(mM)	role	GPL reaction	PPE reaction
none (control)			100	100
DTT	1	TGase stabilizer	100	80
	5		98	21
iodoacetamide	1	TGase inhibitor	0	100
	5		0	88
p-HMBA	1	TGase inhibitor	0	100
	5		0	88
o-phenanthroline	1	DAO inhibitor	91	4
	5		82	0
iproniazide	1	DAO inhibitor	101	12
	5		104	0
semicarbazide	1	DAO inhibitor	100	0
	5		105	0

[a] The residual amine-binding level is expressed as percentage of the bound amine molecules, measured in the absence of chemicals added (1526 nmol of putrescine/mg of substrate × mg of endogenous protein for PPE and 10 071 nmol of putrescine/mg of substrate × mg of endogenous protein for GPL TGase). Reaction conditions: 75 μL of PPE or 30 μL of TFC (*ca.* 100 and 15 pkat of enzyme as TGase, respectively) first incubated during 20 min at 4 °C with the indicated concentrations of the different compounds. The remaining activity was determined after 4 h of incubation at 37 °C, as described in Table 2.

Although animal TGases are usually protected by reducing agents, because of their SH group in the active site (Folk and Cole, 1966), dithiotreitol (DTT) did not increase the level of amine bound to proteins in the PPE-catalyzed reaction; 5 mM DTT even led to a loss of almost 80% of the amine-binding activity (Table 3). These results are in agreement with those published by Icekson and Apelbaum (1987) who found that 1.5 mM DTT induced a 40% inhibition of the pea meristem amine binding. Moreover, sulfhydryl-alkylating reagents like iodoacetamide and *p*-hydroxymercuribenzoic acid (*p*-HMBA) (1 mM), which totally inactivated GPL enzyme, did not significantly modify PPE amine-binding activity (Table 3). Even after incubation with these alkylating agents at 5 mM, more than 80% of the initial binding PPE activity was recovered.

On the other hand, when specific inhibitors of DAO were employed, almost all amine-binding activity of PPE was lost. Addition of 1 mM *o*-phenanthroline, a copper ion-complexing agent, completely inactivated the enzymatic extract. In the same way, compounds like iproniazide and semicarbazide, which block carbonyl groups (Clarke *et al.*, 1959), totally reduced the putrescine-binding activity of pea seedling enzyme but had no effect on GPL TGase (Table 3).

DISCUSSION

According to Icekson and Apelbaum (1987), a great amine-binding activity was found in the supernatant of pea seedling extract, indicating that the active proteins are mainly of cytoplasmic origin. As no activity was recovered in the extract prepared from the first extraction pellets using detergents, we can conclude that in

the case of pea seedlings, the enzyme responsible for the amine binding is not related to membrane proteins as was described in lupine (*L. albus*) seedling extract (Siepaïo and Meunier, 1995) and green tissues of alfalfa (*Medicago sativa*) (Margosiak *et al.*, 1990).

By the PPE extraction procedure, the proteins extracted are mainly of cytoplasmic origin. Depending on the authors and on the plant material used, the type of enzyme responsible for this amine binding was designated as TGase (Icekson and Apelbaum, 1987; Margosiak *et al.*, 1990), TGase-like (Grandi *et al.*, 1992; Aribaud *et al.*, 1995), and DAO (Siepaïo and Meunier, 1995). However, their potentialities regarding deamidation or protein cross-linking have never been described or investigated.

The lower incorporation of amine and the less sensitivity to the acylation when the reaction was catalyzed by the PPE may indicate that the plant enzyme specificity is quite different from that of animal TGases or catalyzes the amine binding in a different way. The ammonia release during the reaction catalyzed by GPL TGase was slightly higher than the quantity of amine incorporated, indicating that among the glutaminyl residues, most of them act as amine acceptors and some of them as water acceptors. The proportion of glutamine being deamidated varied with the substrate, but the results are comparable to those of Mycek *et al.* (1959) who obtained 2.5 times more ammonia liberated than putrescine bound.

Using PPE, the quantity of ammonia released is incompatible with the content of amidated residues of the protein. On the basis of TGase mechanism, the amount of NH_3 released would correspond to the deamination of 30 glutamines/mol of casein, which is impossible according to casein amino acid composition (average value: 17 glutamines/mol of total casein). Using GPL TGase, only 3 glutamines/mol of casein was deamidated. The corresponding values for glycinin were 374 mol of NH_3 using PPE and 20 mol of NH_3 using GPL TGase compared to an average value of 288 glutamines/mol of glycinin. These results suggest that the amount of ammonia released could not be related to the glutamine residues but to the putrescine oxidation into aldehyde by DAO. In these two substrate conditions, polymerization was observed, especially in the PPE-catalyzed reaction. The fact that no polymers were obtained in the absence of amine suggests a prominent part of the amine in the polymer formation. Moreover the nature of the added amine influenced the level of polymerization (Figure 3), the better amine substrates for DAO (Pec *et al.*, 1991) leading to a higher level of polymerization by PPE.

In other respects, the absence of ammonia released when external amine was omitted in the PPE-catalyzed reaction indicated that contrary to GPL TGase, the PPE extract was not able to catalyze any deamidation (Table 2).

CONCLUSION

All the results obtained in the present study suggest that pea seedling cross-linking and amine-binding activities are not related to an acyltransferase activity like TGase but to an oxidative activity like DAO. They also give evidence for the ability of the enzymatic extract (PPE) to cross-link proteins in the presence of added diamines, which was never shown for DAO. The comparative effects of TGase and DAO inhibitors on the reactions catalyzed by PPE indicated clearly that DAO was present in the extract and not TGase. These results led us to conclude that amine binding was catalyzed by DAO and that the cross-linking may also be due to a DAO. Further studies have been performed using purified pea seedling DAO to confirm these results.

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Received for review March 25, 1996. Revised manuscript received July 22, 1996. Accepted August 20, 1996.[⊗]

JF960201F

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1996.