Pea Seedling Extracts Catalyze Protein Amine Binding and Protein Cross-Linking. 2. Contribution of Diamine Oxidase to These Reactions

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The activity of a purified pea diamine oxidase (DAO) was compared to that of a pea seedling enzymatic extract (PPE). It has been shown that DAO is able to catalyze as PPE the binding of amine to proteins as well as protein cross-linking reactions, but only if diamines and Cu^{2+} ions are both available in the medium. We proposed a mechanism in two steps for this reaction: first, an enzymatic step which induces the formation of an aldehyde resulting from the enzymatic oxidation of the diamine by DAO and, second, a chemical step catalyzed by Cu^{2+} which corresponds to the addition of nucleophilic amino acid side chain with the carbonyl group of the aldehyde. This last chemical reaction may lead in a first stage to the amine binding or in a second stage to protein cross-linking.

Keywords: *Pea seedling diamine oxidase; amine binding; cross-linking; deamidation; Pisum sativum*

INTRODUCTION

The diamine oxidases (DAO, EC 1.4.3.6) play a major role during development, ageing, and senescence of cells through the amine metabolism by catalyzing the oxidative deamination of their primary amino groups into aldehydes according to the general reaction:

$$R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + H_2O_2 + NH_3$$

They have been detected for many years in various plant tissues from legumes species like pea seedlings (Hill, 1971), pea cotyledons or leaves (Srivastava *et al.*, 1981), or soybean radicles (Tajima *et al.*, 1985) and also in cereal embryos (Chaudhuri and Ghosh, 1984) and tubers of *Helianthus tuberosus* (Torrigiani *et al.*, 1989).

Pea DAO, purified from epicotyls, is a copper-containing enzyme characterized by a molecular weight around 180 kDa and composed of two polypeptides of 85 kDa linked by disulfide bridges (Yanagisawa *et al.*, 1981). It has been shown to belong, as do some other oxidoreductases, to the group of quinoproteins which use a pyrroquinoline (PQQ) as a cofactor (Glatz *et al.*, 1987).

Only a few studies are concerned with the reaction of the oxidation products of the DAO with proteins. Williams-Ashman and Canellakis (1980) have, however, speculated that these aldehyde compounds could react with amino groups into proteins as they do with nucleic acids. The amine derivative could be incorporated into a protein as a Schiff base (Lorand and Conrad, 1984). DAO was never described as catalyzing protein polymerization reactions, whereas we claimed in part 1 of this paper that this enzyme should play a major role in polymerization reactions when diamine is added. In the present study, we wish to confirm this role of DAO by using pea seedling DAO purified according to Hill (1971) and to propose a reaction mechanism.

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MATERIALS AND METHODS

Diamine Oxidase (DAO). DAO was purified from epicotyls (shoots and apical meristems) of 10 days aged pea seedlings according to the procedure described by Hill (1971). Its activity was measured by the method of Frydman *et al.* (1987). The principle of this spectrophotometric technique is that the pyrroline formed after putrescine oxidation reacts with *o*-aminobenzaldehyde to release a quinazolium salt which absorbs at 435 nm. The enzyme activity was expressed in nkat; 1 kat is defined as the amount of enzyme that oxidizes 1 mol of putrescine/s under experimental conditions. Specific activity was expressed as the activity/mg of proteins in the pea enzyme extract.

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).

RESULTS

Protein Modifications Induced by Purified Pea Seedling DAO. The activity of this purified DAO for catalyzing the reactions of putrescine oxidation, NH_3 release, amine binding, and casein cross-linking was compared to that of PPE (Figures 1 and 2). As was expected, the formation of putrescine oxidation products (pyrroline and NH_3) was largely increased when purified DAO was employed (Figure 1a,b). On the contrary, amine-binding ability of purified DAO was drastically decreased (about 90%) compared to that of PPE (Figure 1c). In the same way, purified DAO was not able to

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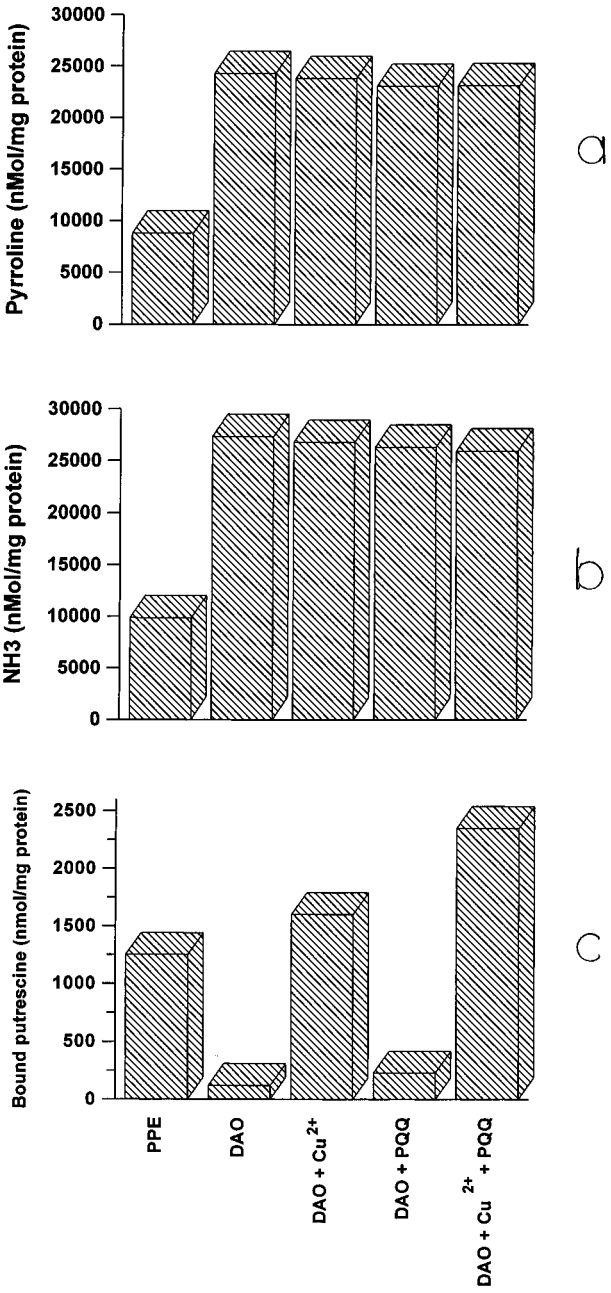


Figure 1. PPE- and purified pea seedling DAO-catalyzed reactions of putrescine oxidation (a), NH_3 liberation (b), and putrescine binding to casein (c). Results are expressed in nmol of product/mg of enzymatic preparation protein. Reaction conditions: 75 μL of PPE (*ca.* 0.35 nkat as DAO activity) or purified DAO (9 nkat as DAO activity) alone or previously incubated with indicated amounts of cofactors (Cu^{2+}, 0.5 mM; PQQ, 0.5 mM) at 4 °C for 60 min and then incubated at 37 °C for 2 h with 1 mg of added native casein, 40 mM cold putrescine, 200 nCi of [^{14}C]putrescine, and 100 mM sodium citrate buffer (pH 6.5), in a final volume of 150 μL.

cross-link casein (Figure 2, lane 3), as did the PPE extract (Figure 2, lane 2).

As observed in earlier experiments (part 1), the amine-binding and cross-linking activities were lost during purification steps like ultrafiltration, dialysis, or size exclusion chromatography. Taking into account these results, we suspected that a cofactor, essential to the amine-binding reaction but not for the putrescine oxidative deamination, could be lost.

Pea seedling DAO needs copper ions (Smith, 1985) and pyrroloquinoline quinone (PQQ) (Glatz, 1987) as cofactors. Normally they are firmly bound to the

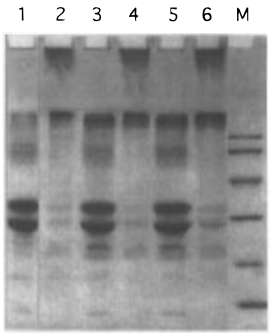


Figure 2. PPE- and DAO-catalyzed cross-linking of casein in the presence of putrescine. Four hour reaction products were submitted to SDS−PAGE (20 μg of protein loaded): lane 1, control reaction, without enzyme addition; lane 2, PPE-catalyzed reaction; lane 3, DAO-catalyzed reaction; lane 4, DAO-catalyzed reaction with 0.5 mM Cu^{2+} addition; lane 5, DAO-catalyzed reaction with 0.5 mM PQQ addition; lane 6: DAO catalyzed reaction with 0.5 mM Cu^{2+} and 0.5 mM PQQ addition; lane M, molecular weight protein markers (94, 67, 43, 30, 20, and 14.4 kDa, from the top). Reaction conditions: the same as listed in Figure 1.

enzyme (Kluetz *et al.*, 1980), but their concentration could be decreased by the purification procedure. When these cofactors were added to the reaction mixture, no increase of the oxidative deamination products was observed (Figure 1a,b). This means that the remaining quantity of cofactors associated with the purified enzyme is high enough to insure the oxidation reaction. However as shown in Figure 1c, the amount of amine bound to casein in the DAO-catalyzed reaction was increased 14 or 20 times, after addition of 0.5 mM Cu^{2+} or 0.5 mM of Cu^{2+} + 0.5 mM of PQQ, respectively.

By comparing the amount of bound putrescine (about 2500 nmol/mg of protein) to the whole oxidized putrescine measured as pyrroline (about 25 000 nmol), it appears that only a minor part (10%) of the oxidized putrescine participates in the amine-binding/cross-linking reaction. The major part of γ-aminobutyraldehyde spontaneously condenses into pyrroline.

Addition of Cu^{2+}, alone or with PQQ, also restored the cross-linking activity of DAO as shown by electrophoresis (Figure 2, lanes 4 and 6). Whereas with DAO alone, no polymerization was observed, in the presence of Cu^{2+} the reaction catalyzed by this enzyme led to the formation of polymers. This point has never been pointed out before.

DISCUSSION

DAO has clearly been shown in the present study to catalyze amine binding and cross-linking of proteins, but the reactions are strongly dependent of Cu^{2+} concentration as proved by our experiments with purified pea DAO. However, the cross-linking mechanism is not yet clear. According to the literature DAO is known to catalyze the oxidation of diamines into aldehydes as described in Figure 3. Aldehydes are very reactive molecules which could participate indeed in protein cross-linking reactions. In fact, the best-known aldehyde cross-linking example occurs in collagen and elastin as a result of oxidative deamination catalyzed by lysyl oxidases of lysine and δ-hydroxylysine (Feeney and Whitaker, 1988). These aldehydes react spontaneously with another aldehyde residue (aldol condensation) or with amino groups (aldimine reaction) to form intramolecular and intermolecular cross-links. In the present study, the formation of Schiff bases between the

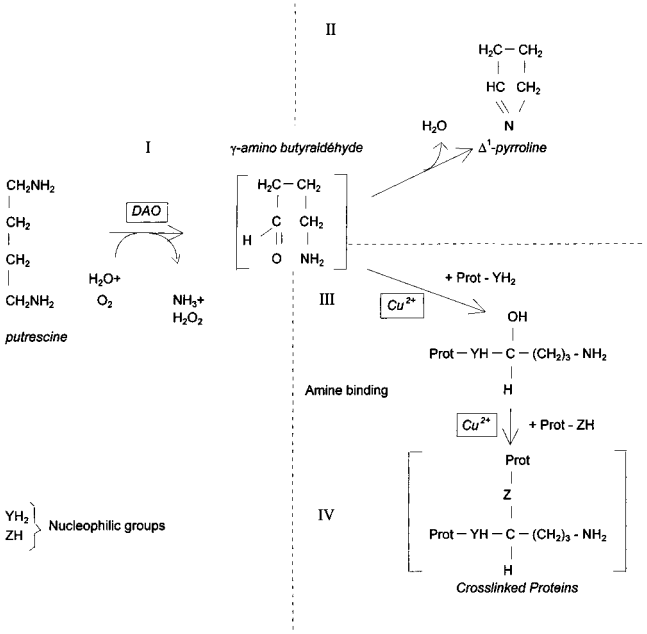
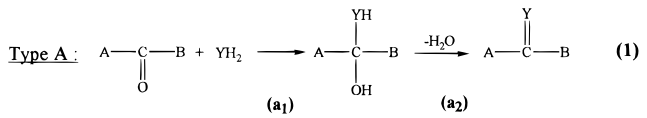
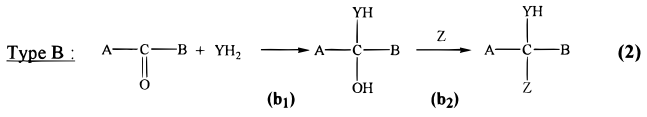


Figure 3. Proposed mechanism for amine-binding and cross-linking reactions dependent on DAO activity: I, enzymatic putrescine oxidation reaction; II, spontaneous condensation of γ-aminobutyraldehyde into pyrroline; III, Cu^{2+} dependent protein cross-linking reaction with γ-aminobutyraldehyde.

generated aldehydes and the lysyl residues of the proteins should also occur; that may explain the amine binding but not the cross-linking between the polypeptide chains nor the strong reactivity of acylated proteins, characterized by lysyl residues blocked. Cross-linking might be explained through the oxidation of diamines into bifunctional reagents; it is well known, however, that diamine oxidases react with only one of the two amino groups of the diamine and are not consequently able to generate bifunctional molecules. This literature background related to our own results led us to propose another mechanism to explain the cross-linking activity and the great reactivity of acylated proteins taking into account the major role of Cu^{2+} ions.

According to Feeney *et al.* (1975), reactions of carbonyl groups usually involve additions to the carbon−oxygen double bond. Most of them are of two types:





In type A, the adduct loses water and the net result of the reaction is the substitution of C=Y−R for C=O. Strongly basic amines react according to type A reactions. Weak bases such as amides or secondary amines undergo reactions according to type B; in this case there is rapid substitution, the hydroxyl group of the tetrahedral intermediate being replaced by a Z group which can be another YH moiety. Many of the addition reactions to the carbon−oxygen double bond are subjected to acid and base catalysis. Bases can catalyze the reaction by converting a reagent of the form YH_2 to the more powerful nucleophile YH^-. Acids may catalyze the reaction by protonating the oxygen atom of the carbonyl compound, making its carbon atom a much stronger electrophile. Similar catalysis may be found with metal ions, such as Cu^{2+}, which may act as Lewis acids. These ions can decrease the electron density at the carbonyl carbon, increasing its reactivity for a nucleophilic group addition.

Such a mechanism could be used to explain the Cu^{2+} dependent DAO cross-linking activity considering that the carbonyl compound corresponds to the aldehyde produced by the amine enzymatic oxidation and the nucleophilic reagent (Y-NH_2) to protein side chains. We consequently propose the reaction mechanism summarized in Figure 3. The first step of the reaction is enzymatic; the DAO catalyzes the oxidation of putrescine into γ-aminobutyraldehyde (Figure 3, I). The major part of this aldehyde condenses itself into pyrroline (Figure 3, II), according to the type A addition reaction. The second step is dependent on Cu^{2+}; a minor part of γ-aminobutyraldehyde (*ca.* 10%) is linked by its carbonyl group to a nucleophilic group of the protein catalyzed by Cu^{2+} (Figure 3, III). In this reaction, many amino acid side chains, like ϵ-NH_2 of lysyl residues, imidazole group of histidine, or guanidinium of arginine, can react as strong basic amines according to the type A reaction. Reactions of type B can also occur through the amide groups of glutamine or asparagine residues. That explains why amine binding is not affected by the acetylation of the lysyl residues.

The cross-linking of proteins can only be explained by a type b_2 reaction (eq 2) in which the Z component is a second nucleophilic amino acid side chain (Figure 3, IV). The hydroxyl group of the intermediary component (eq 2) can be substituted by amide groups or even hydroxyl groups of serine, threonine, or tyrosine.

This mechanism (Figure 3, reactions I and II) explains that the oxidation of a single amino group of the diamines by DAO into aldehyde can occur without added Cu^{2+} and that the production of ammonia and pyrroline was not influenced by the addition of this ion. It also explains (Figure 3, reactions III and IV) why the amine binding and the polymerization of proteins only occured if Cu^{2+} ions are added. This mechanism is also compatible with the fact that acylated proteins are reactive. Moreover, it clearly appears that Cu^{2+} ions act as catalyzers by decreasing the electron density at the carbonyl carbon, reinforcing its electrophilic character.

CONCLUSION

In conclusion, the DAO activity, present in pea seedling extracts, is able to catalyze amine-binding and cross-linking reactions if diamines and Cu^{2+} ions are both available in the medium. TGase activity was not detected on the pea seedling extract, since DAO inhibitors were able to completely inhibit amine-binding activity. Furthermore, no γ-glutamyllysine was found. The DAO-catalyzed polymerization may result from a two-step reaction: enzymatic (γ-aminobutyraldehyde formation) and chemical (Cu^{2+}-catalyzed addition of nucleophilic amino acid side chains to γ-aminobutyraldehyde). However, further experiments must be done in order to identify more precisely the reactive amino side chains and the chemical structure of the reaction products.

ABREVIATIONS USED

CE, crude extract; DAO, diamine oxidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid tetrasodium salt; GPL, guinea pig liver; Ci; Curie; *p*-HMBA, *p*-(hydroxymercuri)benzoic acid; PITC, phenyl isothiocyanate; pkat, picokatals; POD, peroxidase; PPE, partially purified pea seedling extract; PQQ, pyrroloquinoline quinone; RP HPLC: reversed-phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; TCA, trichloroacetic acid; TGase, transglutaminase; TNBS, 2,4,6-trinitrobenzene sulfonate; UF, ultrafiltration.

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