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INHIBITION OF DIAMINE OXIDASE AND ITS REVERSAL BY A NATURALLY OCCURRING PROTEIN

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Key Word Index—*Pisum sativum*; *Cicer arietinum*; *Lens culinaris*; Leguminosae; diamine oxidase; calmodulin antagonists; trifluoperazine; phenolics; flavonoids; quercetin; regulation.

Abstract—Diamine oxidase (DAO) present in pea, bengal gram and lentil is inhibited by trifluoperazine but not by other calmodulin antagonists and the inhibition is reversed by a heat-stable tissue protein of M_r 60000. This protein can also reverse the inhibition caused by phenolics and thus may have a role in regulating DAO activity in the presence of phenolics.

INTRODUCTION

In an earlier communication [1] we reported that pea diamine oxidase (DAO) activity is phytochrome-mediated, involving calcium as a second messenger since spraying plants with EGTA (a calcium chelator), lanthanum chloride and diltiazem (calcium channel blockers) abolished the red light mediated response. Interaction of calcium in cellular processes could be regulated through calmodulin, an intracellular calcium-binding protein [2]. In plants a number of enzymes such as NAD kinase, Ca-ATPase and protein kinases are known to be activated by calmodulin [3–5]. To investigate the involvement of calmodulin in phytochrome-mediated regulation of DAO activity, calmodulin antagonists like trifluoperazine (TFP), *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W_7) and calmidazolium (compound R_{24571}) were used. Inhibition of enzyme activity by these compounds has largely been interpreted for calmodulin mediation. However, there are reports that they may also act in a non-specific manner [6]. The present paper reports the results of the studies carried out on DAO of pea with these compounds.

RESULTS AND DISCUSSION

Addition of $CaCl_2$ and EGTA to the assay system had no effect on DAO activity (Table 1) indicating that Ca is not required for the catalytic function of the enzyme but may have an indirect role by affecting the synthesis of the enzyme. Addition of calmodulin antagonists TFP, W_7 and R_{24571} to the assay in the absence or presence of Ca showed that only TFP inhibited the enzyme by 80% at 10 μM. The residual activity was, however, not inhibited further even by increasing the TFP concentration to 100 μM. W_7 and R_{24571} did not inhibit the enzyme even at 100 μM. These results indicate that DAO activity may not be calmodulin dependent since TFP could inhibit the enzyme even in the absence of Ca. Calmodulin is known to bind to target enzymes only after its combination with Ca. The Ca–calmodulin complex thus formed, as a result of conformational change, exposes a hydrophobic domain to which TFP binds with high affinity [7]. Also the binding site on calmodulin for both TFP and W_7 are the same [8] and hence both should have inhibited DAO if calmodulin was involved. Both TFP and W_7 have a non-specific effect [6] and inhibition of non Ca-calmodulin mediated enzymes by TFP has also been reported [9]. It thus appears that DAO itself may bind TFP.

A concentration curve for TFP (Fig. 1) showed that it is a very potent inhibitor of DAO with an IC_{50} value of 3 μM. IC_{50} values ranging from 10 to 320 μM have been reported for Ca–calmodulin mediated enzymes by various calmodulin antagonists [9–11]. TFP belongs to the phenothiazine class of calmodulin antagonists, which are cationic and have been used to inhibit cation binding enzymes [9]. Since the substrates for DAO are also cationic amines it is possible that TFP may compete for the substrate binding site. However, a substrate saturation curve in the absence or presence of various concentrations of TFP when plotted as double reciprocal plot (Fig. 2) showed that K_m (1.25 μM) for putrescine as substrate was not affected by TFP but V_{max} was significantly decreased indicating that TFP acts as a non-competitive inhibitor (K_i 2 nM). The inhibitory effect of TFP was completely removed when TFP- treated enzyme was dialysed, indicating a weak and reversible interaction of the enzyme with TFP (data not shown).

Since TFP has a very high affinity for calmodulin, it was of interest to see if addition of calmodulin would reverse the inhibition of DAO by TFP. A crude calmodulin preparation free from DAO activity obtained from

Table 1. Effect of EGTA and calmodulin antagonists on DAO activity

Additions	Concentration (μM)	DAO activity (%)
—	—	100
EGTA	100	100
TFP	10	20
	100	20
W_7	10	100
	100	95
R_{24571}	10	100
	100	100

The DAO activity was identical in the absence or presence of 5 mM Ca^{2+}.

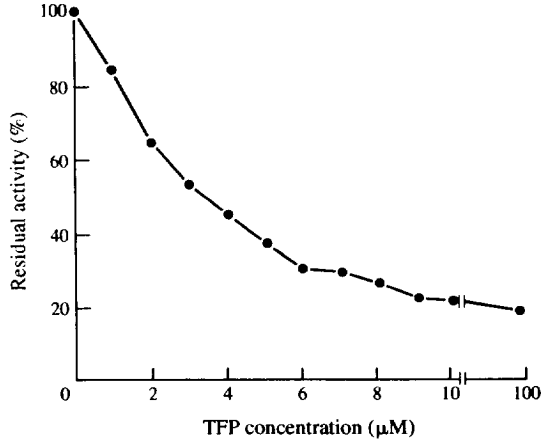

Fig. 1. Effect of TFP on DAO activity *in vitro*.

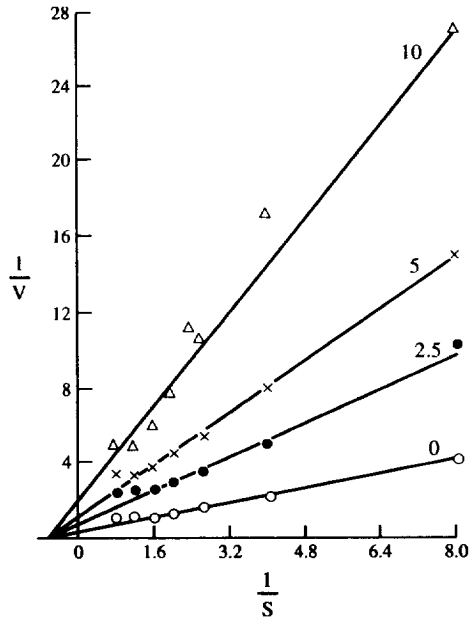

Fig. 2. Double reciprocal plot of DAO activity vs putrescine concentration at various TFP concentrations. TFP concentrations (μM) are indicated on the lines.

Table 2. Effect of calmodulin on inhibition of DAO by TFP and quercetin

Additions	DAO activity (%)
—	100
TFP	30
TFP + CaM (crude)*	98
TFP + CaM (pure)†	30
Quercetin	53
Quercetin + CaM (crude)*	98
Quercetin + CaM (pure)†	53

*Crude calmodulin (1 ml) from pea terminal buds was used.
†Pure bovine brain calmodulin (2 μg) was used.
TFP and quercetin were used at a concentration of 10 and 100 μM, respectively.

pea terminal buds was used. Addition of this calmodulin fraction to the assay system containing TFP could completely reverse the inhibition of TFP (Table 2). This reversal was not affected by the absence or presence of Ca. However, pure bovine calmodulin could not reverse the TFP inhibition either in the absence or presence of Ca. These data suggested that crude calmodulin preparation has some other factor which reverses the inhibition of DAO by TFP. In an earlier study it was shown that the crude calmodulin preparation from pea and bovine brain calmodulin are equally effective on Ca-calmodulin dependent NAD kinase from pea [12]. The protein nature of the factor present in crude calmodulin fraction was evident from the fact that it was non-dialysable, though heat-stable, and treatment with trypsin abolished its ability to reverse the inhibition of DAO by TFP. The protein factor further purified by FPLC had a M_r of 60 000. Two micrograms of this protein could completely reverse the inhibition of DAO by TFP (data not shown).

Our earlier studies have shown that pea cotyledon DAO is inhibited by a number of phenolics and flavonoids, quercetin being the most active [13]. A number of flavonoids such as catechin, epicatechin and quercetin have been reported to be calmodulin inhibitors [14]. Since quercetin has a conjugated aromatic ring structure, it was of interest to see if inhibition of DAO activity by quercetin could also be reversed by the protein factor. The results showed that the inhibition of quercetin was reversed by the crude calmodulin preparation but not by pure bovine brain calmodulin (Table 2). Two micrograms of purified protein factor in the assay was sufficient to reverse the quercetin inhibition.

TFP could inhibit the DAO activity of bengal gram and lentil seedlings also. These seedlings also had a protein factor similar to that of pea which could reverse the TFP and quercetin inhibition and the protein factor of these seedlings was interchangeable (data not shown).

Since TFP is not a naturally occurring compound the question arises about the role of this protein factor. It appears that the inhibition of DAO by phenolics and flavonoids may be regulated by this protein. Quercetin is one of the major flavones of pea [15] and a number of phenolics and flavonoid binding proteins exist *in vivo* [16]. This protein may thus have a regulatory role in controlling DAO activity in the presence of phenolics and flavonoids.

EXPERIMENTAL

Plant material. Pea (*Pisum sativum* L.), bengal gram (*Cicer arietinum* L.) and lentil (*Lens culinaris* L.) seeds were soaked in H_2O for 8 hr and germinated in moistened vermiculite for 6 days in the dark at 25°.

Prepn of homogenate. Terminal buds of etiolated seedlings were homogenized in a chilled mortar and pestle with 0.1 M Tris–HCl buffer (pH 7).

Purification of pea DAO and protein factor. Pea terminal buds were ground in a chilled mortar with cold Me_2CO (− 10°). The dried Me_2CO powder was extracted with 0.1 M Tris–HCl buffer (pH 7) in a chilled pestle and mortar. The suspension was centrifuged at 5000 *g* for 10 min and the supernatant was loaded on calcium phosphate gel in a gel: supernatant ratio of 3:5. The unadsorbed fraction was discarded. Both DAO and the protein factor were adsorbed on gel. DAO was eluted by using 0.2 M Na-Pi buffer (pH 7) and the protein factor by using 0.4 M Na-Pi buffer (pH 7). Both eluates were extensively dialysed against H_2O. The 0.2 M fraction was loaded on DEAE–cellulose equilibrated with 0.01 M Na-Pi buffer (pH 7). The enzyme was not adsorbed and came out in the unadsorbed fr. This fr. had a sp. act. of 1 μkat mg^{-1} and gave a 10-fold enrichment.

The 0.4 M eluate after dialysis was concd to one-tenth of the original vol. by ultrafiltration with a Pm 10 membrane. This was then loaded on FPLC column. A M_r standard was run with alcohol dehydrogenase (M_r 150000) bovine serum albumin (M_r 66000) carbonic anhydrase (M_r 29000) and cytochrome *c* (M_r 12400).

Enzyme assay. DAO activity was assayed as described in ref. [17].

Prepn of crude calmodulin. Crude calmodulin from pea terminal buds was prepared as described in ref. [12].

Treatment of protein factor with trypsin. The protein factor was incubated with trypsin (1 μg ml^{-1}) for 1 hr at 37°. The tube was then heated at 90° for 4 min to inactivate trypsin activity before using it for reversal studies.

Protein estimation. Protein content was determined according to ref. [18].

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