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THE PURIFICATION OF CYTOKININ OXIDASE FROM *ZEA MAYS* KERNELS

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Key Word Index—*Zea mays*; Gramineae; sweetcorn kernels; cytokinin oxidase; cytokinin metabolism.

Abstract—Cytokinin oxidase has been purified to apparent homogeneity from *Zea mays* kernels as indicated by a single protein staining spot on a 2-D, IEF/SDS PAGE gel. Polyclonal antibodies raised to this protein were able to precipitate cytokinin oxidase activity from a highly active, partially purified (QAE-Sephadex) preparation in the presence of fixed *Staphylococcus aureus* cells. The polyclonal antibodies raised to cytokinin oxidase from *Z. mays* cross-react with a similar protein in partially purified cytokinin oxidase preparations from dried wheat seeds.

INTRODUCTION

The metabolism of externally applied cytokinins in plant tissues is generally quite complex. However, in the case of cytokinins having a Δ^{-2} unsaturated side chain, side chain cleavage resulting in loss of cytokinin activity is relatively common, e.g. in *Zea mays* tissues [1, 2] *Vinca rosea* crown gall tissue [3] and in cytokinin-dependent tobacco cell suspensions [4]. The possibility that side-chain cleavage may be a major mechanism for regulating endogenous cytokinin levels has prompted several investigations into the nature of the enzyme responsible for this reaction.

The presence of an enzyme capable of catalysing the cleavage of the N^6 side-chain was first shown in cultured tobacco tissue [5], and was subsequently partially purified from *Z. mays* corn kernels [6]. Further attempts to purify and characterise 'cytokinin oxidase' have since been made by a number of workers; a M_r of 88 000 was determined for the enzyme from *Z. mays* kernels [6]. Whitty and Hall [6] established the enzyme requirement for molecular oxygen and the presence of a Δ^{-2} double bond in the side-chain of the substrate. Brownlee *et al.* [7] showed that when IP was the substrate the enzyme produced adenine and 3-methyl-2-butenal. It was postulated by Whitty and Hall [6] and Brownlee *et al.* [7], and subsequently confirmed by Laloue and Fox [8], that an unstable imine intermediate is formed during the reaction.

Laloue and Fox [8] found that a partially purified cytokinin oxidase preparation from wheat germ was strongly inhibited by the cytokinin active ureas, diphenyl urea and *N*-(2-chloro-4-pyridyl)-*N'*-phenyl urea, a result that was confirmed in the course of this work using a partially purified enzyme preparation from *Z. mays* kernels (Burch and Horgan, unpublished results).

In *Phaseolus vulgaris* callus tissue, cytokinin oxidase activity could be induced by the external application of cytokinin and the urea derivative Thidiazuron, the latter also inhibited the activity of partially purified cytokinin oxidase from this tissue [9]. *In vitro* enzyme activity was also shown to be enhanced in the presence of copper-imidazole complexes, even in the absence of molecular oxygen, which suggested that the complexes were substituting for molecular oxygen in some way [10].

Comparative work on the substrate specificity of partially purified cytokinin oxidase from *Z. mays* kernels and *V. rosea* crown gall tissue has been carried out in which both enzymes were only active on substrates with a Δ^{-2} double bond in the side chain, although large side chain substituents made the cytokinin resistant to both oxidases. The *Vinca* enzyme appeared to differ from the *Zea* enzyme only in terms of its M_r, 25 000 (*V. rosea*) compared to 94 000 (*Z. mays*) [11].

The strong probability that cytokinin oxidase is also able to regulate cytokinin levels in plants has prompted us to initiate studies into the nature and regulation of this enzyme in a range of plant tissues. In order to do this, it was initially necessary to completely purify the enzyme, to raise polyclonal antibodies to a single protein.

In the present study, we have purified a protein with cytokinin oxidase activity from *Z. mays* kernels. We have raised polyclonal antibodies to this protein which also cross-reacts with a similar protein in partially purified cytokinin oxidase preparations from dried wheat seeds.

RESULTS

Cytokinin oxidase purification from 1700 g of *Z. mays* kernels is summarized in Table 1. Significant removal of the bulk of interfering proteins was achieved in all steps, as shown in Fig. 4, although this was not always reflected in the specific activity values. This was presumed to be mainly due to a loss of activity through the latter half of the procedure, and because for convenience sake the enzyme was often assayed under non-optimal conditions. However, the main objective was to obtain a pure protein for immunological purposes and so the purification was monitored by SDS-PAGE. Cytokinin oxidase activity was not readily detected in the crude buffer extract of *Zea* kernels, but was highly active in a 40–60% ammonium sulphate precipitate (previously found to be the most

Abbreviations: FPLC, fast protein liquid chromatography; IP, isopentenyladenine.

Table 1. Purification of cytokinin oxidase, starting from 1700 g of *Z. mays* kernels

	Total protein (mg)	(Sp. activity mg/protein hr)	Increase in specific activity
60% $(NH_4)_2SO_4$ ppt.	4.0×10^3	0.12	—
QAE-Sephadex	1.42×10^3	0.16	1.4 ×
MONO-Q; FPLC	0.232×10^2	1.15	9.7 ×
Phenyl superose; FPLC	0.745×10	1.14	9.7 ×
Concanavalin-A Sepharose 4B	0.15	100.80	850 ×
Bio-sil TSK-125 Gel filtration	0.048	69.2	590 ×

active fraction [11]). The following chromatographic procedures used to further purify the 60% ammonium sulphate precipitate are shown in Figs 1–3. Significant purification was achieved with each of these chromatographic steps (see 12% SDS-PAGE gel, Fig. 4), however, it was not until the Con-A step that it was possible to try to associate enzyme activity with a particular band on a gel. The high degree of purification achieved on Con-A Sepharose was in agreement with the findings of Chatfield [12], who identified cytokinin oxidase as a glycoprotein and first used Con-A sepharose to partially purify the enzyme.

The methyl mannopyranoside eluate when examined by SDS-PAGE showed the presence of a high M_r protein (78 000) and four lower M_r components (32 000; 29 000; 18 000 and 14 000). The relatively large M_r differences between each group of proteins meant that their separation could be achieved by high resolution gel filtration, an important technique in that we were unable to recover enzyme activity from the gels. The UV elution profile and cytokinin oxidase activity of the combined and concentrated methyl mannopyranoside eluate from Con-A Sepharose, when chromatographed on a Bio-Sil TSK 125 column are shown in Fig. 3. Cytokinin oxidase activity was associated with a peak eluting at an approximate M_r of 44 000; SDS-PAGE of four successive fractions across this peak revealed that the activity was associated with a protein of M_r 78 000 (Fig. 5).

Approximately 100 μg of purified protein was obtained from 1.7 kg of *Z. mays*; with no effort being made to

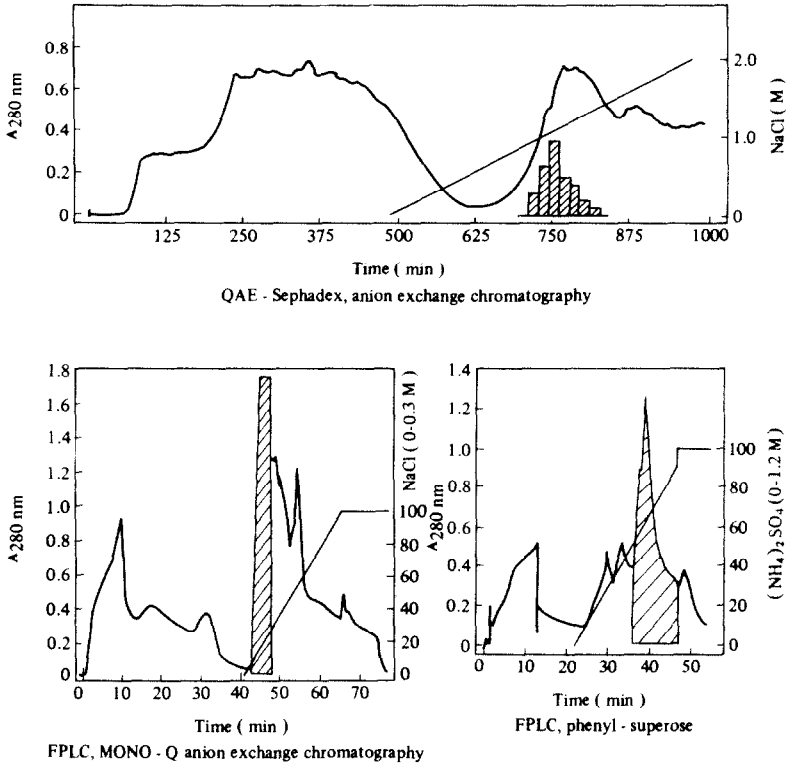

Fig. 1. UV absorbance and cytokinin oxidase activity elution profiles for the initial stages of cytokinin oxidase purification from *Z. mays* kernels. The shaded areas represent the eluate fractions with significant oxidase activity which were used for subsequent steps.

optimise yield, recovery was low (estimated to be *ca* 0.7%). The homogeneity of the combined active fractions from the TSK 125 column was assessed by 2-D IEF/SDS PAGE (Fig. 6), the results of which indicated that the active fractions contained a single protein. The purified protein was used to raise polyclonal antibodies in a rabbit. Purified IgG showed significantly enhanced immunoreactivity towards both a purified cytokinin oxidase preparation (TSK 125) from *Z. mays* and to a relatively impure wheat enzyme preparation, when compared to non-immune serum control. This was tested by dot blotting using a gold labelled goat anti-rabbit second antibody procedure with silver enhancement.

Significantly, the antibody preparation was able to precipitate the cytokinin oxidase activity of a highly active, partially purified (QAE-Sephadex) preparation from *Z. mays* kernels in the presence of fixed *Staphylococcus aureus* cells.

A Western blot analysis of a purified cytokinin oxidase preparation from *Z. mays* (Fig. 7; lanes 1 and 2) and a partially purified preparation from dry wheat seeds (Fig. 7; lane 3) showed that although raised to a native protein, the cytokinin oxidase anti-bodies recognised an

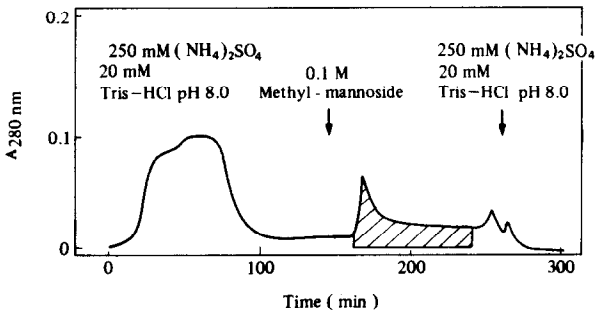

Fig. 2. Concanavalin-A sepharose 4B affinity chromatography of Phenyl superose FPLC fraction shown in Fig. 1. The shaded area represents cytokinin oxidase activity which was collected in nine 4 ml fractions.

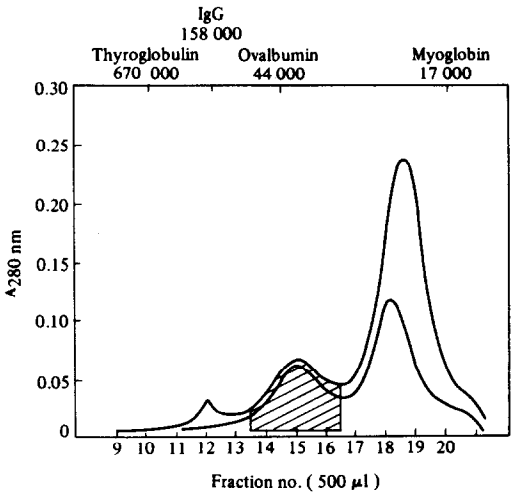

Fig. 3. Elution profile of combined and concentrated cytokinin oxidase active fractions from Con-A Sepharose 4B after gel filtration on a Bio-Sil TSK 125 column. Cytokinin oxidase activity was located mainly in fractions 14 and 15, but was also present in fractions 13 and 16.

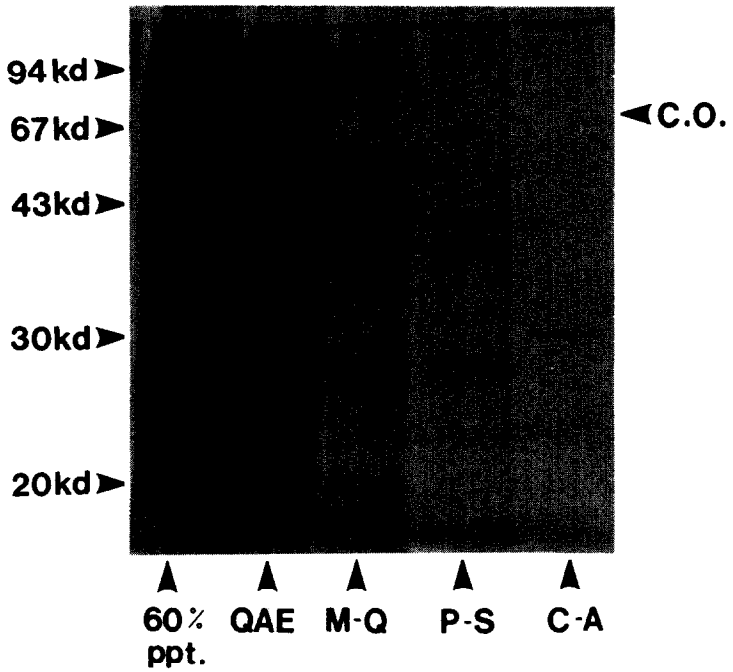

Fig. 4. 12% SDS-PAGE gel showing samples containing cytokinin oxidase activity from each purification step, indicating that significant purification was achieved with each of these chromatographic steps.

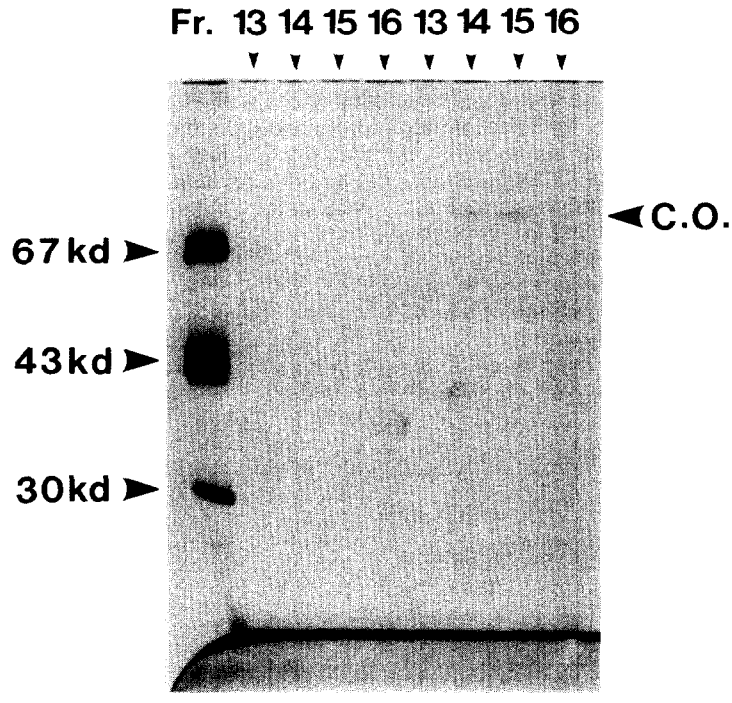

Fig. 5. 10% SDS-PAGE gel of samples containing cytokinin oxidase activity from Bio-Sil TSK 125. Enzyme activity was proportional to the intensity of staining of the protein band in each lane.

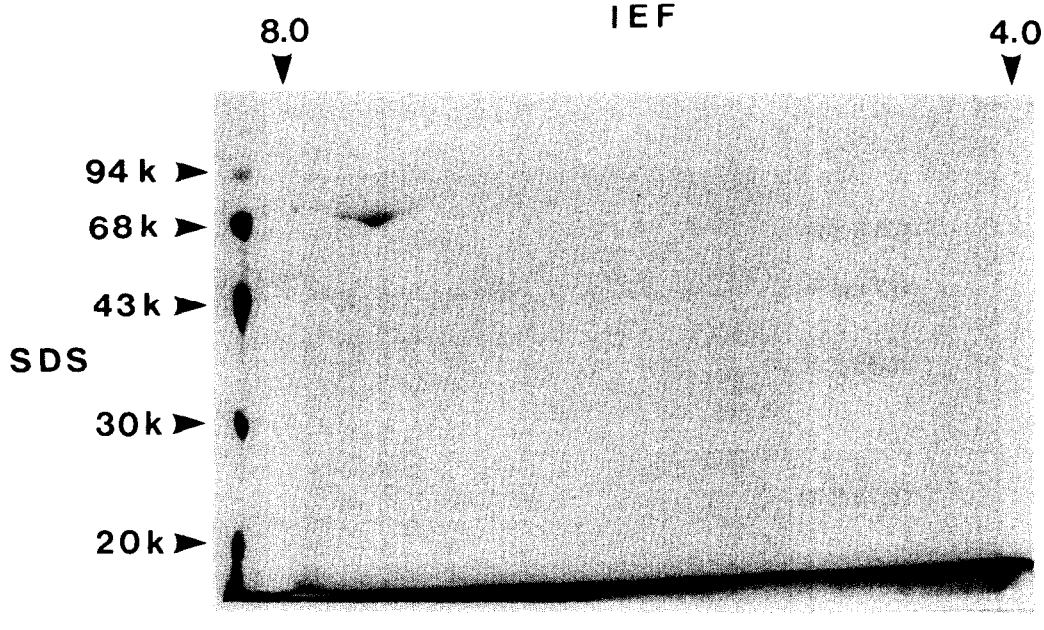

Fig. 6. 2-D IEF/SDS PAGE analysis of the combined TSK 125 fractions 14 and 15 from Fig. 5. Approximately 3 μg of protein was loaded onto the IEF gel.

SDS denatured protein from *Z. mays* and also cross-reacted with a similar protein from dry wheat seeds.

DISCUSSION

Cytokinin oxidase from *Z. mays* kernels was purified to apparent homogeneity using the method described in this paper. However, when the same method was applied to the purification of the enzyme from wheat, it was much less successful. Figure 7 shows the purification achieved in the final two steps; the methyl mannopyranoside eluate from Concanavalin-A Sepharose 4B indicated that wheat seeds contain a far larger number of glycoproteins that are purified together with cytokinin oxidase. Even so,

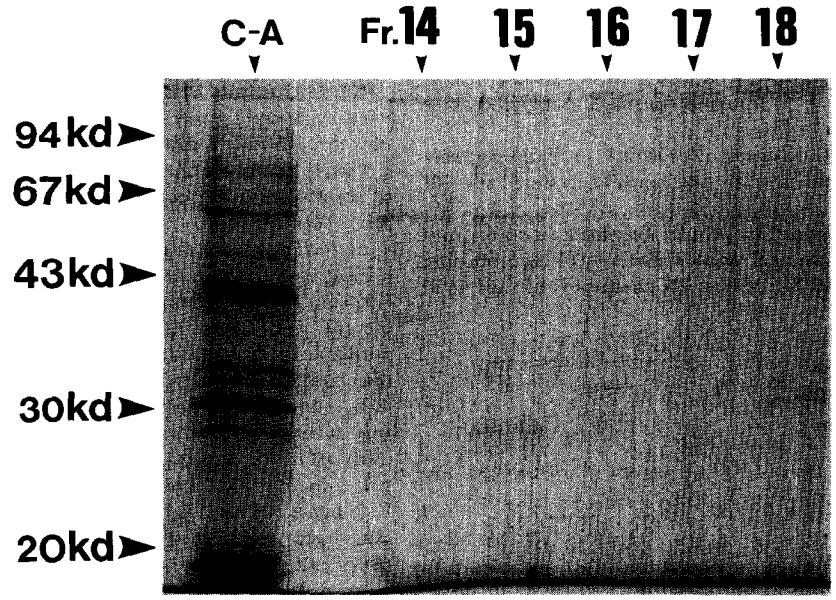

Fig. 7. 12% SDS-PAGE gel showing a partially purified cytokinin oxidase preparation from dried wheat seeds (cv. Avalon). The purification scheme described in the Experimental was used to purify the wheat enzyme. The gel shows the purification achieved in the final two steps; Concanavalin-A Sepharose 4B (C-A), and fractions 14 to 18 from Bio-Sil TSK 125 gel filtration.

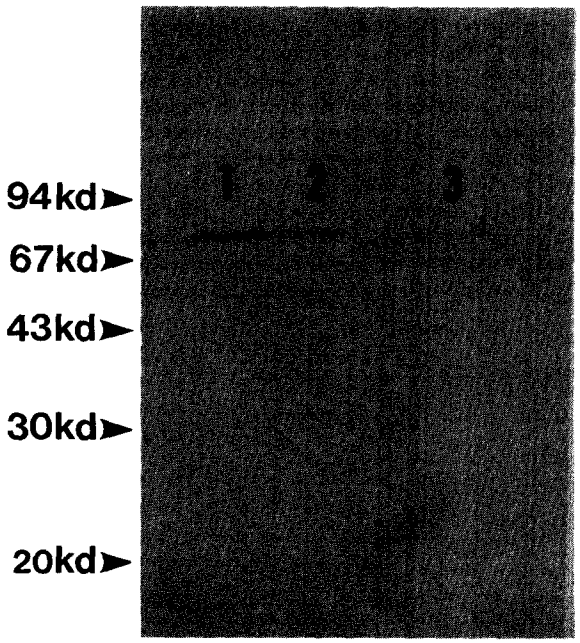

Fig. 8. Western blot using antibodies to *Z. mays* cytokinin oxidase against a purified enzyme preparation from *Z. mays* kernels (lanes 1 and 2) and a partially purified preparation (TSK 125, fr. 15) from dried wheat seeds (lane 3). Rabbit IgG was detected by gold labelled goat anti-rabbit second antibody with silver enhancement.

Fig. 8 shows that in a complex mixture of wheat glycoproteins, the antibody raised to *Z. mays* cytokinin oxidase will recognise a protein of the same size in wheat seeds, and we therefore conclude that the cytokinin oxidase from wheat germ, studied by Laloue and Fox [8] is very similar to the *Z. mays* enzyme. This work is also aimed at determining the exact nature of the enzyme, including factors that may regulate its activity *in vivo*. It has been shown that an imine intermediate is formed during the reaction catalysed by cytokinin oxidase [8]; thus mechanistically cytokinin oxidase would appear to be a copper-dependent amine oxidase. We tested this directly after it was suggested that copper-dependent amine oxidases may catalyse their own inactivation by recognition of amino-acetonitrile as a substrate [13]. The results shown in Fig. 9 indicate that *Z. mays* cytokinin oxidase was susceptible to amino-acetonitrile induced inactivation.

The copper-dependent amine oxidases have been reported to have an additional prosthetic group, possibly pyridoxal phosphate [14], although the enzyme from pea seedlings (the only example from plants reviewed by Malmstrom *et al.* [15]), did not seem to contain pyridoxal phosphate, and therefore another cofactor may be present. The loss of the suggested cofactor, or loss of copper itself, may have been the cause of the apparent loss of activity observed in the latter stages of the purification of cytokinin oxidase from *Z. mays* kernels.

A group of compounds that have been shown to be cytokinin active and have also been shown to regulate cytokinin oxidase activity *in vitro* [8, 9] are the diphenyl ureas and their derivatives. Structurally, it is difficult to propose the diphenyl ureas as cytokinin analogues and preliminary competition studies using partially purified (Mono-Q, FPLC) *Z. mays* cytokinin oxidase, indicated that they may act as non-competitive inhibitors of the enzyme (Fig. 10).

In conclusion, the use of immunoaffinity chromatography will enable us to obtain quantities of pure protein with which to establish the exact nature of the enzyme, and the antibodies raised to cytokinin oxidase will also be used to determine the presence of the enzyme in other plants.

EXPERIMENTAL

Chemicals. All the chemicals used were obtained commercially; [8-^{14}C]IP (433 MBq/mmol) was made using procedures of ref. [16].

Plant materials. Cobs of corn (*Zea mays* L. cv. Earliking) were obtained from a local merchant and immediately stored at −20° until required.

Assay of cytokinin oxidase activity. Enzyme assays were routinely carried out with total reaction mixture (115 μl), containing 100 μl of enzyme (in 20 mM Tris–HCl buffer, pH 8.0) and 15 μl [8-^{14}C]IP, at 37° for 4–6 hr. The reaction was stopped by the addition of 30 μl $HClO_4$ and the mixture was centrifuged briefly (MSE, Micro-Centaur) before the reaction products were analysed by reversed phase HPLC (ODS-Spherosorb) linked to a flow through radioactivity monitor (Reeve Analytical).

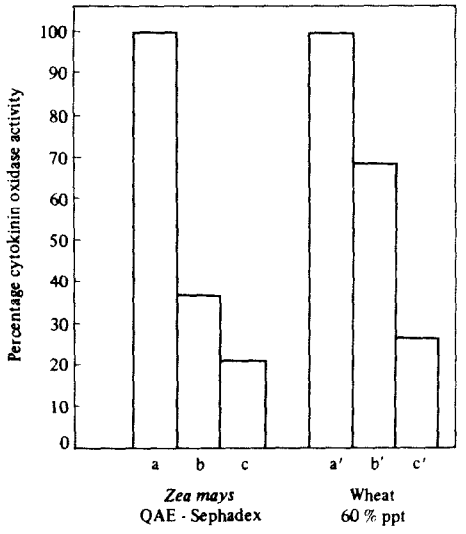

Fig. 9. Bar graph showing the inhibition of both partially purified *Z. mays* and wheat cytokinin oxidase preparations by aminoacetonitrile. a,a': control, 100% activity, b,b': reaction mixture containing 5 mM aminoacetonitrile., c,c': reaction mixture containing 50 mM aminoacetonitrile. Assay conditions were as described in Experimental.

Protein assays. Protein was either determined on the basis of its absorbance at 280 nm or by the protein-dye binding assay of ref. [17].

Extraction and purification of the corn enzyme. Corn kernels (1700 g) were homogenized in 0.1 M K-Pi buffer, pH 6.8 (1500 ml) containing insoluble polyvinylpyrrolidone (50 g) and ascorbic acid (10 g). The homogenate was filtered through muslin and then centrifuged at 10000 *g* (1.5 hr). The pellet was discarded and solid $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation and centrifuged at 10000 *g* (1.5 hr). The pellet was retained and the supernatant was brought to 60% $(NH_4)_2SO_4$ satn and again centrifuged at 10000 *g* (1.5 hr). The ppts from both $(NH_4)_2SO_4$ steps were resuspended in 0.002 M K-Pi buffer, pH 6.8 (150 ml) and dialysed against 2 × 2.0 l of the same buffer overnight at 4°. The dialysates were centrifuged at 40000 *g* (1.5 hr) and the supernatant stored at −20°. *Preparative anion-exchange chromatography.* The 40–60% $(NH_4)_2SO_4$ ppt. (180 ml) was applied to a QAE-Sephadex column (3 × 40 cm) (60 ml extract applied per run), equilibrated with 20 mM Tris–HCl buffer, pH 8.0. Cytokinin oxidase activity was eluted from the column in a gradient of NaCl (0–2.0 M). Fractions containing enzyme activity were combined and dialysed against 2 × 2.0 l 20 mM Tris–HCl buffer pH 8.0 overnight. *Analytical anion-exchange chromatography.* Dialysed, combined fractions from the QAE-Sephadex step were applied onto an FPLC Mono-Q (Pharmacia, HR 5/5) column, equilibrated with 20 mM Tris–HCl, pH 8.0. Cytokinin oxidase activity was eluted from the column in a gradient of NaCl (0–0.3 M). *Hydrophobic interaction chromatography.* Fractions containing cytokinin oxidase activity from Mono-Q FPLC were combined and brought to a concn of 1.2 M $(NH_4)_2SO_4$ by addition of 3.0 M salt (i.e. 4 ml 3.0 M $(NH_4)_2SO_4$ added to 6 ml Mono-Q fraction). This was applied to a Phenyl Superose FPLC column (Pharmacia, HR 5/5), equilibrated with 1.2 M $(NH_4)_2SO_4$ in 20 mM Tris–HCl pH 8.0. Cytokinin oxidase activity was eluted in a gradient of 100–0% 1.2 M

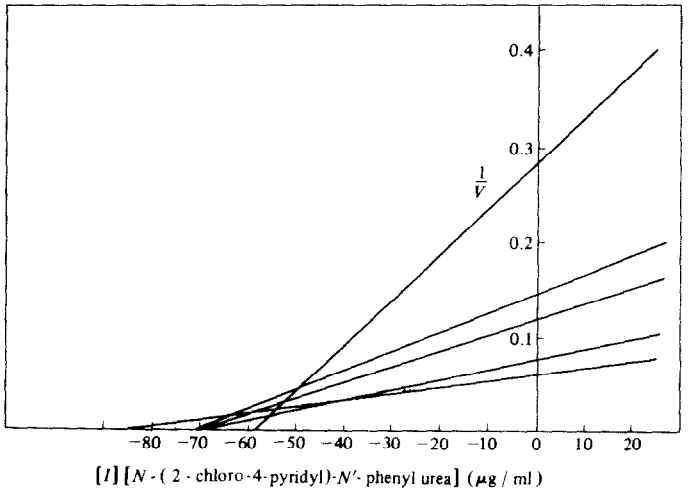

Fig. 10. Dixon plot of cytokinin oxidase activity as a function of inhibitor concentration. Assay was performed with Mono-Q FPLC partially purified enzyme, in 10 mM potassium phosphate buffer, pH 6.8. Reaction mixtures contained between 5 and 60 μl stock [8-^{14}C]IP and were incubated at 37° for 2 hr. Lines were fitted by regression analysis.

$(NH_4)_2SO_4$. *Concanavalin-A Sepharose 4B.* Active fractions from the previous chromatography step were pooled and loaded onto a concanavalin-A Sepharose 4B column (1 × 6 cm) (Sigma), equilibrated with 0.25 M $(NH_4)_2SO_4$ in 20 mM Tris–HCl pH 8.0. Cytokinin oxidase activity was eluted from the column in a wash of equilibration buffer containing 0.1 M methyl-mannopyranoside. *Gel filtration chromatography.* The fractions eluted from Con-A by methyl-mannopyranoside were concentrated down to a vol of 500 μl by pressure filtration (Sartorius, Ultrasart Cell 10) and 5 × 100 μl were loaded onto a Bio-Sil TSK 125 gel filtration column (Bio-Rad, 300 × 7.5 mm), equilibrated with 0.1 M NaOAc buffer pH 6.0. Two main UV absorbing peaks were well separated and collected. The smaller, high $-M_r$ peak, was associated with cytokinin oxidase activity. *PAGE.* A silver stained 12% SDS-PAGE gel indicated that the higher M_r peak from the gel filtration step was a single protein band of M_r *ca* 78 000. The purity of the protein was subsequently confirmed by 2-D, IEF/SDS PAGE. Rabbit IgG raised to the pure protein was purified by the caprylic acid method of ref. [18].

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