Phytochemistry, Vol. 22, No. 9, pp. 1885–1889, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00

CHARACTERIZATION OF WHEAT *o*-DIPHENOLASE ISOENZYME

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(*Revised received* 21 *January* 1983)

Key Word Index—*Triticum aestivum*; Gramineae; wheat; *o*-diphenolase; isoenzyme; MW; copper content; amino acid composition; kinetic properties; inhibitors.

Abstract—A highly purified isoenzyme of wheat *o*-diphenolase was characterized. The isoenzyme had a MW of *ca* 115 000, as determined by Sephadex G-100 gel filtration. The copper content was 0.20%, and the amino acid composition was determined. Two subunits (MWs *ca* 30 000 and 23 500) were detected by SDS gel electrophoresis. The K_m was found to be 5.1 mM for 4-methylcatechol and kinetic analysis showed that the isoenzyme exhibited substrate inhibition. The isoenzyme was characterized by its response to some inhibitors.

INTRODUCTION

o-Diphenolase (*o*-DPO) (*o*-diphenol:O_2 oxidoreductase, EC 1.14.18.1) is a copper containing oxidase able to catalyse *o*-hydroxylation of monophenols and dehydrogenation of *o*-diphenols. Although the enzyme from higher plants has been studied extensively, only a few details are available on wheat *o*-DPO.

The isolation and purification of *o*-DPO is difficult [1] and several methods have been reported with varying degrees of success. In two earlier publications [2, 3] we described a procedure based on the isolation of the enzymically most active fraction and on the elimination of the less active ones, together with the impurities. By this method we obtained a wheat isoenzyme which represented a 1750-fold purification over the starting material. More recently, we also compared some properties of common and durum wheat *o*-DPOs [4]. As reports were lacking on a full characterization of the wheat enzyme, a more detailed study seemed justified. The fraction used was the isoenzyme obtained by column isoelectric focusing [3] and it will be characterized as regards MW, copper content, amino acid composition, kinetic properties and effect of inhibitors.

RESULTS AND DISCUSSION

The isoenzyme studied here was obtained as described in the Experimental. An unusual feature was its very high purification and high isoelectric point (9.60).

Molecular weight

An estimate of the MW of the isoenzyme was made after filtration through Sephadex G-100. The distribution of protein and *o*-DPO activity revealed a single component, thus indicating the homogeneity of the isoenzyme with respect to MW. The calculated elution parameters were plotted on the selectivity curve for the Sephadex column and a MW of *ca* 115 000 was estimated. Crude or partially purified phenolase systems from various plants can be separated by gel filtration into several enzymatically active forms with different MWs. Some such forms showed a MW close to that of the wheat isoenzyme studied here. This occurred for apple fruit [5], sugar cane [6] and potato tubers [7]. More recently, Anosike and Ayaebene [8] when studying polyphenoloxidase from yam tubers determined, by gel filtration, the same MW which we have estimated for the wheat *o*-DPO isoenzyme. By gel filtration and ultracentrifugation different MWs have been obtained for enzymatic forms isolated from grapes [9, 10], green olives [11], tea [12], spinach beet [13], bananas [14] and potatoes [15]. However, comparison with MWs of *o*-DPOs from other higher plants is not always exact, either because the methods adopted for MW determination are in many cases different or because the samples were crude or partially purified preparations. In contrast, our isoenzyme shows homogeneity and a high degree of purification.

In general, *o*-DPOs from higher plants can be accounted for in terms of aggregates of monomers. The wheat isoenzyme submitted to analytical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) showed two subunits, one corresponding to a MW of 30 000 and the other to a MW of 23 500 (average from three runs). The ratio of the amount of protein present in the two bands was estimated by comparing the peak areas obtained after scanning the gels. In three different experiments, ratios between 2.6 and 2.8 were obtained. This distribution indicated that 72–74% of the total mass corresponded to the 30 000 MW subunit, whereas 28–26% could be assigned to the 23 500 MW subunit.

These results seem to indicate that the band with MW 30 000 represents a basic subunit of the wheat *o*-DPO, whereas the nature of the weak band with MW 23 500 is less clear. The functional subunit of plant *o*-DPO has frequently been indicated with MW 30 000–40 000 [7, 10, 13, 16]. However, the problem of the monomeric subunit of *o*-DPO from higher plants is not yet resolved. The correlation between the MW and the number of subunits is affected by association–dissociation pheno-

mena [1, 17, 18] and proteolytic degradation of the enzyme during purification [1]. By comparing the MWs of the subunits with the results of the gel filtration experiment, we can hypothesize that the 115 000 MW experimentally observed might be a tetramer formed by four peptide chains, three of MW 30 000 and one of 23 500.

Copper content

It is well-known that wheat *o*-DPO contains copper as its active prosthetic group. Our isoenzyme was found to contain 0.20% total copper. This value agrees strikingly with the copper content of fungi and bacteria diphenolases [19–26], whereas in higher plants the comparison is less significant owing to the small amount of published data. We determined a copper content slightly higher than that obtained from broad beans [27] and potatoes [15] and sharply lower than that from tea [12]. The copper content from grape is exceptionally low [10] but the authors suppose a significant loss during purification. Assuming a MW of 115 000 estimated by Sephadex gel filtration, the purified wheat isoenzyme contained 3.6 atoms of copper per molecule of enzyme, thus giving a copper content of somewhat less than one atom per mol of monomer of MW 30 000, which seems to be the basic unit of wheat *o*-DPO isolated here. It is possible that a small part of the copper was lost during the purification procedure, particularly on the DEAE-cellulose column. The presence of one atom per subunit has been amply documented in fungi, particularly mushrooms and *Neurospora* [19–22], bacteria [25] and in the few reports on higher plants [15, 27]. Some differences can be appreciated in the MW of a subunit rather than in the number of copper atoms. Subsequent studies on mushroom and *Neurospora* diphenolases showed that the enzyme contained a copper pair per functional unit [23, 26, 28]. The problem of the activity and the number of copper atoms of the subunits is still not resolved [1]. The presence of either one or two copper ions per functional unit is of great importance for the reaction mechanism of this enzyme.

The oxidation state of the copper in the enzyme from various sources is uncertain. ESR spectroscopic analysis was carried out to know the valence state of copper in the wheat isoenzyme. From the peak height of the spectrum it is estimated that less than 3% of the copper in the sample was in the cupric state. Therefore, it would seem likely that there is one atom of copper per subunit, probably univalent, since the enzyme appears to be diamagnetic. In reality the problem is more complex. Two cupric atoms might be spin-coupled in such a way as to produce no ESR signal. This topic is discussed in more detail in reviews [1, 29] and specific reports on mushroom and *Neurospora* diphenolases [23, 26, 30, 31], whereas no information about the oxidation state of the copper in the enzyme from higher plants is available.

Amino acid composition

The amino acid analysis data presented in Table 1 gives further support to the present characterization of the wheat isoenzyme.

The basic (lysine, arginine, histidine) and hydrophobic (proline, valine, isoleucine, leucine, phenylalanine) residue contents were 11.4 and 27.7%, respectively. That of sulphur amino acids was 3.0%. Glutamic acid was the most abundant, followed by aspartic acid and glycine. The amino acid composition observed does not account for the high isoelectric point (9.60) of the isoenzyme. However, the values reported for aspartic and glutamic acids include asparagine and glutamine. Until the amide content of the enzyme is known, no conclusions can be drawn. In comparing the amino acid composition of wheat *o*-DPO with that of grape, spinach, beet and potato tuber, calculated on the basis of a polypeptide chain reduced for all the enzymes to MW 10 000 (Table 1), the only other plant diphenolases for which an amino acid composition has been reported [10, 13, 15], it becomes evident that, although there is a similarity of composition, there are also some significant differences. The wheat *o*-DPO showed a basic residue content closely resembling that of the other three sources, whereas the number of hydrophobic residues was relatively lower. The composition of the sulphur containing amino acids compares well with the one reported for potato and spinach enzyme, while in grape the enzyme apparently contained only traces. Furthermore, the wheat enzyme is distinguished by the greater residue content of glutamic acid, glycine, serine and alanine.

Table 1. Amino acid composition of wheat and other plants *o*-DPOs (residues per 10 000 MW)

Amino acid	Wheat (our results)	Grape [10]	Spinach beet [13]	Potato [15]
Lysine	5.2	4.5	4.5	5.6
Histidine	1.7	1.5	2.0	1.7
Arginine	3.5	2.8	2.5	3.3
Aspartic acid	9.4	9.8	10.3	9.2
Threonine	5.2	4.5	4.8	4.7
Serine	7.7	6.0	6.0	6.1
Glutamic acid	10.4	7.0	4.5	8.6
Proline	6.3	6.5	7.0	4.7
Glycine	8.3	5.5	6.5	6.9
Alanine	7.7	6.3	5.8	6.7
Half-cystine	1.4	0.1–0.3	1.8	1.4
Valine	5.6	4.5	6.5	5.8
Methionine	1.4	—	1.8	1.7
Isoleucine	3.5	4.3	3.5	4.7
Leucine	6.6	5.3	7.5	7.8
Tyrosine	3.5	4.5	2.0–2.3	2.8–3.1
Phenylalanine	3.5	3.0	4.3	3.9
Tryptophan	1.0	N.D.	0.5	N.D.
MW	115 000*	80 000†	40 000‡	36 000§

*From gel filtration.
†From sedimentation equilibrium.
‡From SDS electrophoresis.
§From amino acid values.
N.D., Not detected.

The comparison between wheat and fungal *o*-DPOs shows that the amino acid composition is somewhat strikingly different [20, 21, 32, 33].

Kinetic properties

The relative activity of the wheat *o*-DPO towards different mono-, di- and polyphenols, using the fraction

recovered from chromatography on a DEAE-cellulose column, was previously studied [2]. We showed that the enzyme possessed high catecholase activity but very little cresolase activity and that it had the highest activity towards 4-methylcatechol (4MC) as substrate. In this study we used a more purified sample for testing the change in the rate of oxidation with 4MC concentration. When reciprocals of the rates were plotted against the reciprocal of diphenol concentration, the pattern of Fig. 1 was obtained. The reaction showed a marked inhibition at substrate concentration above 10 mM. This characteristic effect is rather unusual in rate studies on *o*-DPOs from various sources. Only a few reports referred to the inhibition of the enzyme at high substrate concentration. The phenomenon was observed in enzymatic preparations, at different purification degrees and with different substrates, from tea [12], potato [34], cherries [35, 36] and banana [37].

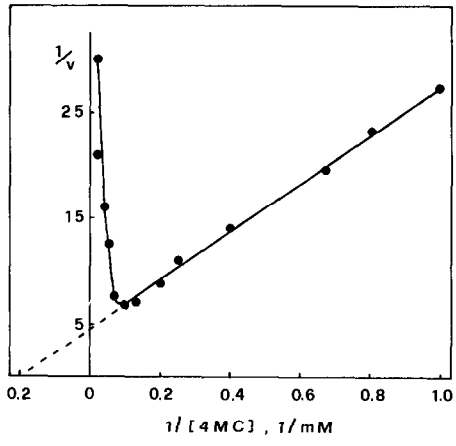

Fig. 1. Lineweaver–Burk plot of the wheat *o*-DPO isoenzyme with 4-methylcatechol as the variable substrate

The apparent Michaelis constant value for 4MC was calculated by extrapolation of the approximatively linear part of the Lineweaver–Burk plots of Fig. 1. The K_m value was found to be 5.13 mM and it was significant only at a 4MC concentration below 10 mM. The K_m indicates that the isoenzyme has a relatively low affinity for 4MC and is in general agreement with the K_m values of plant diphenolases, whereas it is higher when compared with the enzyme from fungi [1].

The percentage inhibition at concentrations of 4MC above 10 mM cannot be directly calculated, since the inhibitor in this case is itself a substrate. However, by comparing the experimental reaction rate curve with that calculated theoretically, the inhibition of wheat *o*-DPO by increasing concentration of 4MC, may be determined. The theoretical curve represents the reaction rate that would be found in the absence of inhibition and it may be constructed using the equation:

$$v = \frac{V_{max}[4MC]}{K_m + [4MC]}.$$

Such a theoretical curve, calculated from the K_m 5.13 mM and V_{max} 0.222, is shown in Fig. 2, together with the experimentally obtained curve. From the difference

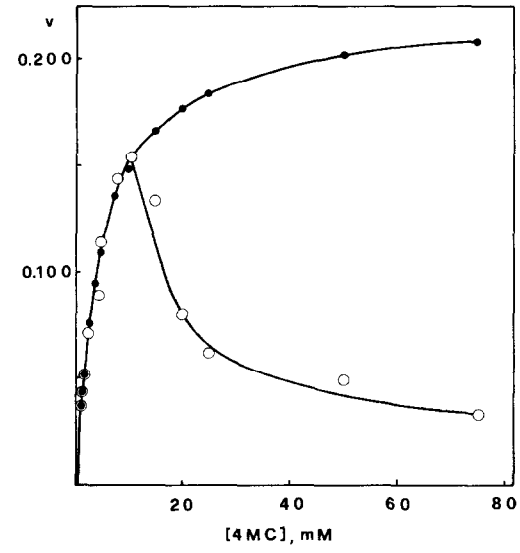

Fig. 2. Effect of 4-methylcatechol concentration on initial rate of wheat *o*-DPO isoenzyme-catalysed reaction. (●) Theoretical data calculated from the K_m and V_{max} obtained from Fig. 1. (○) Experimental data.

between the theoretical and experimental rate values at each concentration, one may calculate the percentage of inhibition as a function of the substrate concentration. From Table 2 it can be seen that in the concentration range 15–75 mM, the percentage of inhibition was *ca* 20–85%.

Effect of inhibitors

Little information is available regarding the nature of substances responsible for the inhibition of wheat *o*-DPO activity. The effect of six inhibitors was studied using a solution (10 mM) of 4MC as substrate. The compounds used are known to be strong inhibitors of plant diphenolases [1]. Pre-incubation of the isoenzyme with the inhibitors caused partial inhibition of the enzyme activity (Table 3). Sodium diethyldithiocarbamate and potassium metabisulfite appeared to strongly inhibit the wheat isoenzyme at a low concentration, while a higher concentration of potassium cyanide and thiourea was needed to obtain an inhibitory effect of 50%. Sodium chloride showed a

Table 2. Percentage inhibition of wheat *o*-DPO isoenzyme as a function of 4-methylcatechol concentration

4MC concn (mM)	$v_t - v_e^*$	Inhibition (%)
15	0.032	19.4
20	0.090	50.8
25	0.121	65.8
50	0.153	76.1
75	0.175	84.1

*Theoretical (v_t) and experimental (v_e) rate values were calculated from the data shown in Fig. 2

Values calculated from the data shown in Fig. 2.

Table 3. The effect of various inhibitors on wheat *o*-DPO isoenzyme

Inhibitor	Concn required for 50% inhibition (mM)
Sodium chloride	5.5×10^{2}
Thiourea	2.3×10^{-1}
Potassium cyanide	1.3×10^{-1}
Potassium metabisulfite	9.2×10^{-2}
Sodium diethyldithiocarbamate	7.2×10^{-2}
PVP	1.1%(w/v)

relatively poor inhibitory effect. The addition of polyvinylpyrrolidone (PVP) to the reaction mixture also depressed *o*-DPO activity.

The response of our isoenzyme to some inhibitors resembles that reported by Singh and Ahlawat [38, 39] for wheat tyrosinase. Appropriate studies are required to clarify the mechanism of inhibition by these compounds.

EXPERIMENTAL

Material. Wheat grains of *Triticum aestivum* (cv Nettuno) were selected for the investigation.

Chemicals. Proteins used as standards for MW determination were purchased from Serva (cytochrome *c*, myoglobin equine, chymotrypsinogen A, bovine pancreas, pepsin porcine, albumin egg, acid phosphatase wheat germ), from Sigma (albumin bovine) and from Boehringer (aldolase rabbit muscle). Sephadex G-100 was obtained from Pharmacia. 4-Methylcatechol (4MC) was supplied by Pfaltz Bauer and was recrystallized from *n*-hexane before use. All other reagents were analytical grade.

Extraction and purification of the enzyme. The *o*-DPO was extracted with 0.05 M NaPi buffer at pH 6.6 and purified by pptn with $(NH_4)_2SO_4$, calcium phosphate gel treatment, ion exchange chromatography and column isoelectric focusing following the procedures previously described [2, 3].

Molecular weight. The approximate MW of the purified isoenzyme was determined by gel filtration on a Sephadex G-100 column, according to the method of ref. [40]. The column (2.5 × 60 cm) was equilibrated and, subsequently, eluted with 0.02 M NaPi buffer, pH 6.2. The elution rate was controlled by a peristaltic pump to 27 ml/hr. Column eluates were continuously monitored for protein by reading the *A* at 280 nm and 2.7 ml fractions collected. Estimation of MW was made by calibrating the Sephadex column with the following proteins of known MWs: cytochrome *c* (12 300), chymotrypsinogen A (25 000), egg albumin (45 000), bovine albumin (67 000), acid phosphatase (85 000), aldolase (158 000). The elution vols. of the markers were determined spectrophotometrically at 280 or 412 nm (cytochrome *c*). The void vol. of the column was taken as the point of elution of Dextran Blue 2000. The elution parameters, K_{av}, were calculated according to ref. [41]. The minimum MW of the isoenzyme was determined by analytical SDS gel electrophoresis [42]. The enzyme was prepared in 0.01 M NaPi buffer, pH 7, containing 1% SDS and 1% 2-mercaptoethanol. Up to 100 μg of the sample, pre-incubated at 50° for 2 hr, was applied to a cylindrical gel (6 × 80 mm). Electrophoresis was carried out at room temp. at 10 mA per gel for 4–6 hr. Gels were stained with Coomassie Blue R-250 for protein, and scanned in a spectrophotometer. A log MW vs electrophoretic mobility standard curve was constructed with bovine albumin (67 000), egg albumin (45 000), pepsin (36 000), myoglobin (17 800) and cytochrome *c* (12 300). Ratios of the mass of protein present in different bands on the same gels were calculated by comparing the peak areas of gel scans.

Determination of Cu. Total Cu was measured in protein samples by atomic absorption analysis. The determination was made in duplicate and the amount of Cu was calculated from a standard curve. ESR spectra were recorded with a Varian ESR spectrometer E-109, using a Cu(II)–EDTA complex as standard.

Amino acid analysis. Amino acids were determined by hydrolysing lyophilized samples in a sealed tube under N_2 with 6 M HCl for 24, 48 and 72 hr at 110°. Hydrolysates were analysed on a Beckman Multichrom B amino acid analyser. Threonine and serine were corrected for destruction during hydrolysis by extrapolation to zero time. The releases of valine and isoleucine were virtually complete after 72 hr, so these values were taken. Two samples were oxidized with performic acid and then hydrolysed for 24 hr to determine the half-cystine content [43]. Our own analyses were conducted on three different preparations of the enzyme. The results obtained were reproducible to ±5% between preparations. Tryptophan content was determined on alkaline hydrolysates with $Ba(OH)_2$[44].

Effect of substrate concentration. o-DPO activity was assayed spectrophotometrically as described in ref. [2]. Concn of 4MC, ranging from 1 to 75 mM in O_2-satd H_2O, was used. K_m and V_{max} values were calculated by least-squares analysis from Lineweaver–Burk plots.

Effect of inhibitors. To examine the effect of inhibitors on *o*-DPO activity, reaction mixtures, containing constant amounts of enzyme and substrate (4MC), were run in the presence of six different inhibitors. The reaction was followed and the rate of reaction was determined according to the method of ref. [2]. The inhibitors were incubated at 25° for 30 min with the enzyme, before the addition of the substrate.

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