candidate for the production of a salt-tolerant food plant through selection, breeding and development of cultivation techniques. Also, improved processing of SM seed, such as more complete hull removal, represents a potential means of improving the nutritional quality of SM seed.

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Studies on the Phenolase Enzymatic System in Durum Wheat

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Ammonium sulfate fractionation of crude preparations of durum wheat (cv. Valnova) *o*-diphenolase, followed by treatment with calcium phosphate gel, ion-exchange chromatography, and column isoelectric focusing, gave an 885-fold increase in specific activity. In the first purification steps, process activation of the enzyme was observed, thus revealing a latent enzymatic activity. The purified enzyme showed activity toward di- and polyphenolic substrates but it did not catalyze the oxidation of monophenols. With 4-methylcatechol as the substrate, the enzyme displayed two pH optima of 5.3 and 7.3. The main maximum was located on a broad plateau of 0.4 pH unit. Isoelectric focusing and ion-exchange chromatography revealed three isoenzymes with different specific activities. Some significant differences between the *o*-diphenolase studied here and that previously isolated by us from a common wheat sample were emphasized.

Browning reactions which occur in some wheat products are believed to be related to the enzyme *o*-diphenolase (*o*-diphenol:O_2 oxidoreductase, EC 1.14.18.1). Besides this well-known function, the enzyme is also believed by some authors to have properties that might be useful in identifying wheat varieties (Lamkin et al., 1981), particularly in the identification of common wheats used in the production of pasta products (Feillet and Kobrehel, 1974; Kobrehel and Feillet, 1976) that in some countries, like France and Italy, is an adulteration. Our previous reports described some properties of *o*-diphenolase isolated from a common wheat sample (Interesse et al., 1980, 1981) and displayed the existence of multiple forms of that enzyme. Wheat isoenzymes have been revealed by many workers using acrylamide gel electrophoresis (Kruger, 1976; Singh and Sheoran, 1972; Taneja et al., 1974; Taneja and Sachar, 1974, 1977a,b; Tikoo et al., 1973). The number of isoenzymes and their properties may be different in each wheat variety. In this study we have isolated and characterized the *o*-diphenolase from a durum wheat sample as well as separated enzymatically active proteins by ion-exchange chromatography and isoelectric focusing. Fur-

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thermore, a comparison between the results obtained here and those observed on the common wheat sample previously investigated (Interesse et al., 1980, 1981) is made.

EXPERIMENTAL SECTION

Sample. Wheat grains of *Triticum durum* (cv. Valnova) were used. Protein content ($N \times 5.7$, dry basis), moisture, and ash contents were 13.3%, 10.7%, and 1.4%, respectively. *o*-Diphenolase was extracted from powdered wheat with 0.05 M phosphate buffer at pH 6.6, and the crude extract (fraction A) was separated.

Purification of the Enzyme. Solid $(NH_4)_2SO_4$ was added to the crude extract solution to 55% saturation. The resulting pellet was isolated by centrifugation, dissolved in phosphate buffer (0.05 M), dialyzed, and centrifuged again. The solution (fraction B) was treated with calcium phosphate gel, and the suspension was then centrifuged, recovering the supernatant designated as fraction C.

Ion-Exchange Chromatography. An aliquot of fraction C (~100 mg of protein corresponding to ~11 500 units of *o*-diphenolase) was loaded on a 2.6 × 13 cm column of DEAE-cellulose. The column was eluted with a linear gradient of phosphate buffer (0.05–0.4 M), pH 7.0, and finally with 0.5 and 1 M NaCl. Fractions (2.8 mL) were collected and assayed for the *o*-diphenolase activity.

Isoelectric Focusing. Final purification of the enzyme was obtained by column isoelectric focusing. Fractions 10–19 (fraction D_1) from two chromatographic runs on the DEAE-cellulose column were pooled and submitted to isoelectric focusing carried out in a LKB electrofocusing column of 110-mL capacity. The density gradient was made up with sucrose, and the pH gradient was established by using Ampholine carrier ampholytes of a pH range 3.5–10.0. Electrofocusing took place for 48 h at a final voltage of 1400 V. Fractions of 2.8 mL each were collected at a rate of 1 mL min^{-1} and assayed for the pH and the enzymatic activity. A separate focusing experiment was performed with the same procedure on another aliquot of fraction C (~60 mg of protein corresponding to ~7000 units of *o*-diphenolase). A detailed description of the purification methods of *o*-diphenolase has been given elsewhere (Interesse et al., 1980, 1981).

Enzymatic Activity on Various Substrates. Tyrosine, protocatechuic acid, caffeic acid, (−)-epicatechin, and chlorogenic acid, purchased from Sigma Chemical Co., and *p*-hydroxycinnamic acid, 4-methylcatechol, and DL-3,4-dihydroxyphenylalanine, from Pfaltz & Bauer, Inc., represented substrates used to detect *o*-diphenolase enzyme. 4-Methylcatechol was recrystallized from *n*-hexane before use. All other chemicals were of analytical reagent grade. Except for tyrosine, the substrate concentration was 0.02 M in phosphate buffer, pH 7.3. Because of its limited solubility, the tyrosine was solubilized until saturation. The wavelengths used for determining the activity toward the various substrates were previously described (Interesse et al., 1980).

Effect of pH. So that the variation in enzymatic activity as a function of pH could be examined, the fraction D_1 eluted from the ion-exchange chromatography column was dialyzed against either 0.1 M citrate–phosphate buffer (pH 4.0–7.0), 0.1 M phosphate buffer (pH 7.0–8.0), or 0.1 M Tris-HCl buffer (higher pH values) before assay.

Assay Methods. *o*-Diphenolase activity was assayed colorimetrically at 395 nm by using 4-methylcatechol as a substrate. The incubation mixture contained 0.5 mL of enzyme solution, 0.5 mL of 0.05 M phosphate buffer, pH 7.3, and 1 mL of 0.02 M substrate solution. Omission of enzyme solution from the assay mixture served as a control.

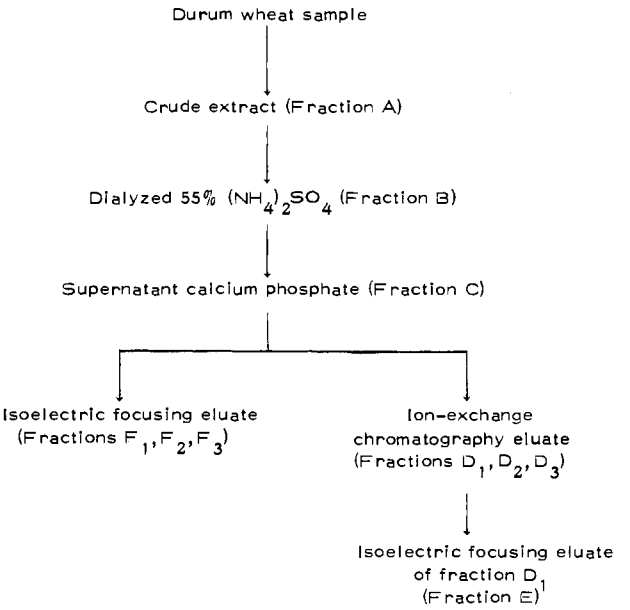

Figure 1. Schematic representation of the fractionation steps of *o*-diphenolase from durum wheat.

The reaction mixture was oxygenated for 5 min prior to the addition of the enzyme extract. One unit of *o*-diphenolase activity was defined as the amount of the enzyme giving a change in absorbance of 0.001 min^{-1} under the conditions used. Protein was estimated by following the procedure of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. Elution of protein in column effluents was continuously monitored by absorbance at 280 nm.

RESULTS AND DISCUSSION

Extraction and Purification. There are many problems associated with the isolation of polyphenol oxidases from plant tissues; these have been reviewed by Mayer and Harel (1979). The scheme that summarizes the purification steps followed in this paper is represented in Figure 1. After the extraction, the ammonium sulfate fractionation together with calcium phosphate gel treatment were employed for a preliminary purification of the enzymatic preparation. The fraction C thus obtained was further purified by means of two different techniques: preparative isoelectric focusing and ion-exchange chromatography on DEAE-cellulose. From both the procedures three enzymatically active fractions have been obtained. The purification was completed by submitting the major fraction eluted from ion-exchange chromatography (fraction D_1) to isoelectric focusing. This procedure yields fraction E, showing *o*-diphenolase activity. Before the properties of durum wheat *o*-diphenolase at various stages of purification were studied its substrate specificity and the pH–activity profile were investigated by using fraction D_1.

Substrate Specificity. *o*-Diphenolases of plant origin are characterized by broad substrate specificity. However, individual enzymes from various sources differ, in general, only quantitatively in their ability to oxidize phenolic compounds. The *o*-diphenolase isolated here was tested for activity with mono-, di-, and polyphenols as substrates. Table I shows the results obtained from the enzymatic assays. 4-Methylcatechol was found to be the best substrate; therefore, the various affinities have been expressed as a percentage of activity toward 4-methylcatechol. The relative activities of durum wheat *o*-diphenolase toward di- and polyphenolic substrates (catecholase activity) are comparable to those previously obtained for the common wheat sample (Interesse et al., 1980). The only appreciable

Table I. Enzyme Specificity toward Different Substrates

substrate	relative act.
monophenols	
tyrosine	0
p-hydroxycinnamic acid	0
diphenols	
4-methylcatechol	100
protocatechuic acid	0
DL-3,4-dihydroxyphenylalanine	55
caffeic acid	35
polyphenols	
(−)-epicatechin	10
chlorogenic acid	20

difference is the relative activity toward epicatechin, which is lower than the activity toward DL-3,4-dihydroxyphenylalanine and similar to that with caffeic acid, whereas for common wheat it was only slightly below the activity toward 4-methylcatechol. Moreover, Table I shows that the isolated *o*-diphenolase has no affinity toward monophenolic compounds (cresolase activity). The preferential oxidation of di- and polyphenols in comparison with monophenols has been observed in *o*-diphenolase of many plants (Benjamin and Montgomery, 1973; Coggon et al., 1973; Coombs et al., 1974; Hasegawa and Maier, 1980; Lanzarini et al., 1972; Rivas and Whitaker, 1973; Wong et al. 1971). The absence of cresolase activity, however, is not a characteristic of the enzyme, but it can probably be attributed to some structural changes that take place during the protein purification that may cause a loss of cresolase activity (Mayer and Harel, 1979) and/or to the fact that the hydroxylation of monophenols occurs after a lag period (Kahn and Pomerantz, 1980).

pH Profile. A plot of *o*-diphenolase activity as a function of pH (Figure 2) gave a curve with two pH optima at 5.3 and 7.3, with the activity at pH 5.3 only 55% of the maximum. A comparison with the pH profile of common wheat *o*-diphenolase (Interesse et al., 1980) shows that the main maximum is 0.4 pH unit shifted toward more alkaline values and that it belongs to a broad plateau between pH 7.3 and pH 7.7. The presence of this plateau is similar to that (pH 7.3–7.8) observed for some fractions of cherry phenolase (Benjamin and Montgomery, 1973). Below pH 7.3, the activity quickly decreases to a value that is 15% of the maximum. Even if the pH optimum of *o*-diphenolase varies with the source from which it is prepared, the presence of two peaks is a common feature of many polyphenol oxidases (Mayer and Harel, 1979).

Properties at Various Purification Steps. *o*-Diphenolase activity was determined at each stage of purification by using the initial velocity of the oxidation reaction of 4-methylcatechol at pH 7.3. A summary of the results obtained is given in Table II.

(*a*) *Ammonium Sulfate Precipitation.* The wheat *o*-diphenolase activity was recovered in the pellet obtained

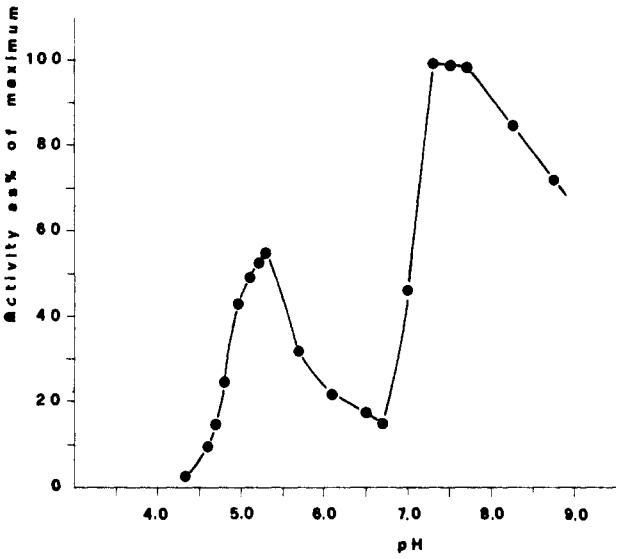

Figure 2. Effect of pH on the activity of durum wheat *o*-diphenolase.

at 55% saturation with ammonium sulfate. The specific activity in the dialyzed fraction (fraction B) was 7-fold higher than that of the crude extract. The increase of total units of activity with a yield greater than 100% (235%) is noteworthy. Once again we must emphasize that the *o*-diphenolase is present in a latent form. This phenomenon has already been observed in common wheat sample and an interpretation has been given previously (Interesse et al., 1980).

(*b*) *Treatment with Calcium Phosphate Gel.* As a result of the calcium phosphate gel treatment the enzymatic activity of fraction C significantly increased, reaching 18625 units, thus yielding the latency phenomenon of the enzymatic system much more evident. The enzyme recovered from the supernatant calcium phosphate treatment was purified 68-fold over the crude extract. The *o*-diphenolase of durum wheat sample at this purification step is less purified than that isolated by the same procedure from common wheat (Interesse et al., 1980).

(*c*) *Ion-Exchange Chromatography.* The partially purified fraction C was passed through a column of DEAE-cellulose, and absorbances at 280 nm were recorded (Figure 3). Three of the proteins (fractions D_1, D_2, and D_3) showed enzymatic activity toward 4-methylcatechol as the substrate. Together they contain ~30% of the protein originally applied to the column. The first peak, obtained after elution with 0.15 M phosphate buffer, contains ~63% of the activity recovered by chromatography. Its high (286.3 units mg^{-1}) specific activity indicated a 168-fold purification over the starting material and 2.5-fold increase over fraction C. The other two fractions showing enzy-

Table II. Purification of *o*-Diphenolase from Durum Wheat

fraction	tube	volume, mL	total protein, mg	enzyme act. units mL^{-1}	enzyme act. total units	sp act., units mg^{-1}	purification
A		90.0	721.38	14	1260	1.7	1
B		76.0	252.35	39	2964	11.7	7
C		74.5	160.11	250	18625	116.3	68
D_1	10-19	28.0	20.93	214	5992	286.3	168
D_2	28-35	22.4	1.95	53	1187	608.7	358
D_3	60-68	25.2	7.84	95	2394	305.3	180
E	34-39	16.8	6.31	565	9492	1504.3	885
F_1	17-20	11.2	16.88	103	1154	68.4	40
F_2	22-26	14.0	9.03	116	1624	179.8	106
F_3	33-40	22.4	7.78	164	3674	472.2	278

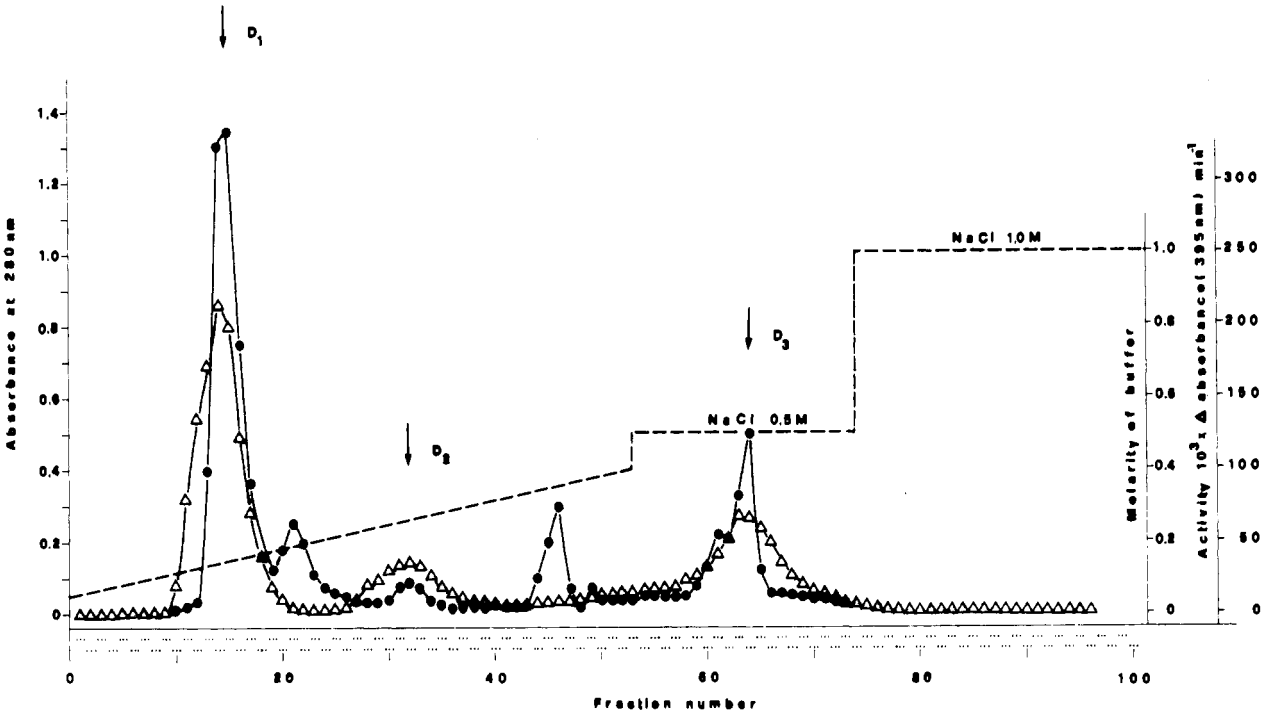

Figure 3. Chromatography on a DEAE-cellulose column of durum wheat *o*-diphenolase eluted with a concentration gradient of phosphate buffer at pH 7.0, then 0.5 M NaCl, and 1.0 M NaCl: (△) *o*-diphenolase activity; (●) absorbance at 280 nm; (---) concentration of the eluting solution.

matic activity (D_2 and D_3) were eluted with 0.26 M phosphate buffer and 0.5 M NaCl, respectively; the properties of the three fractions are summarized in Table II. One can easily observe that ion-exchange chromatography produces on the whole a decrease of the number of total units; therefore, the latency phenomenon does not take place in this purification step. Further studies were conducted on fraction D_1 because of its high yield.

Chromatographic profiles of durum and common (Interesse et al., 1980) wheat were similar. The durum wheat sample shows three enzymatically active proteins, whereas the common wheat sample showed four active fractions, but samples have most of the enzyme concentrated in one single peak eluted at low values of ionic strength.

(*d*) *Column Isoelectric Focusing.* Fraction C was purified with column isoelectric focusing (Figure 4). The pH gradient was linear between pH 3.5 and pH 10.0. Fraction C was resolved into five proteins focused at isoelectric points (p*I*s) of 3.5, 4.9, 5.4, 6.8, and 9.4. Only those focused at p*I*s of 5.4, 6.8, and 9.4 (fractions F_1, F_2, and F_3, respectively) showed *o*-diphenolase activity, and they represent ~56% of the total protein applied to the column. The F_3 fraction focused at alkaline pH and was the most enzymatically active fraction (Table II; Figure 4). The total recovery of the enzymatic activity of the three fractions relative to that of the charged one is ~92%. Fraction F_3 shows a 278-fold purification over the crude extract and 4-fold over the fraction C submitted to the focusing.

A qualitative comparison of results of the isoelectric focusing of durum wheat (Figure 4) with that previously obtained for common wheat (Interesse et al., 1981) shows that durum wheat has a lower number of separated proteins and of enzymatically active fractions than that shown in common wheat. However, the three proteins active toward 4-methylcatechol present in the durum wheat sample are focused at isoelectric points near those of the common wheat sample. One feature of the isoelectric focusing of durum compared with common wheat is the lack of enzymatic protein focused at more acid pH.

The possibility of further purifying the D_1 fraction deriving from the ion-exchange chromatography was investigated. For this purpose, isoelectric focusing was attempted by using the eluates of two chromatographic runs. Figure 5 reports the absorbance and the activity profiles in the pH gradient that was linear between pH 3.5 and pH 10.0. One can observe that several proteins were separated; their isoelectric points can be easily located. However, only one protein shows *o*-diphenolase activity (fraction E); it focuses at more basic pH (p*I* = 9.40). The yield of the enzyme with reference to that charged is ~79%. This isoelectric behavior has already been observed in the common wheat sample (Interesse et al., 1981), thus confirming that the fraction D_1 is still a polydisperse proteic system. The removal of inactive protein forms by isoelectric focusing gives rise to fraction E, which is more purified (5-fold) than fraction D_1, and achieves an 885-fold purification over the crude extract with a final specific activity of 1504.2 units mg^{-1}. However, the specific activity of the durum wheat *o*-diphenolase is always lower than that of common wheat.

Multiple Forms. *o*-Diphenolase like other enzymes possesses molecular heterogeneity (Balasingam and Ferdinand, 1970; Ben-Shalom et al., 1977; Harel et al., 1973; Jolley et al., 1969; Matheis and Belitz, 1975; Montgomery and Sgarbieri, 1975; Pifferi and Cultrera, 1972; Zenin and Park, 1978). Many authors have referred to the molecular multiplicity of this enzyme in wheat (Kruger, 1976; Singh and Sheoran, 1972; Singh and Singh, 1974; Taneja et al., 1974; Taneja and Sachar, 1974, 1977a,b; Tikoo et al., 1973). In a previous paper we identified isoenzyme forms in a common wheat sample (Interesse et al., 1981). The present work extends this study to the multiple forms of a durum wheat sample. For this purpose the purification procedure described in Figure 1 has been designed not only to purify the enzyme but also to isolate isoenzyme forms that are associated with fractions at different purification degrees and having high *o*-diphenolase activity. By the two different separation techniques employed, ion-exchange

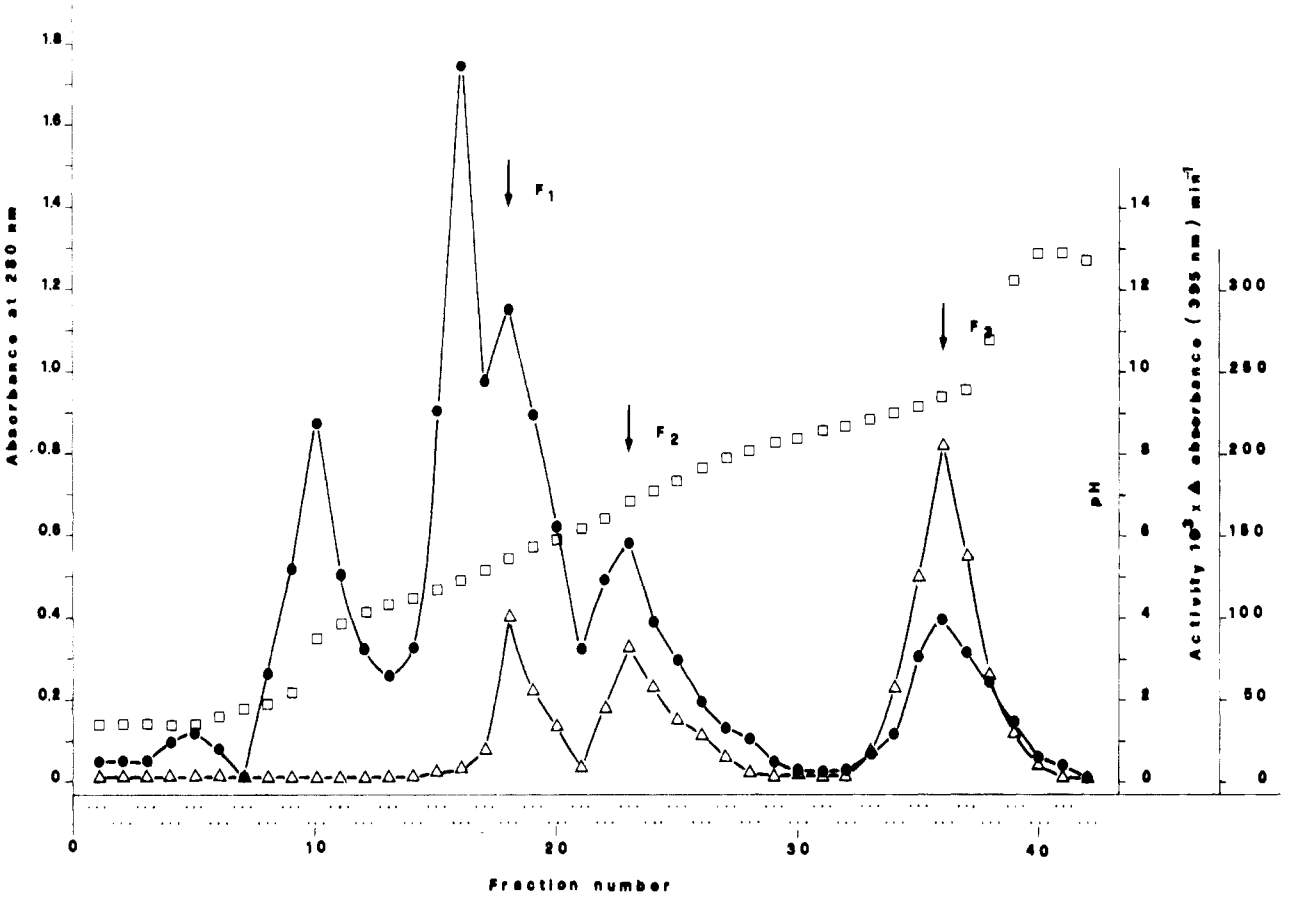

Figure 4. Isoelectric focusing of the fraction C from calcium phosphate gel treatment: (△) *o*-diphenolase activity; (●) absorbance at 280 nm; (□) pH gradient.

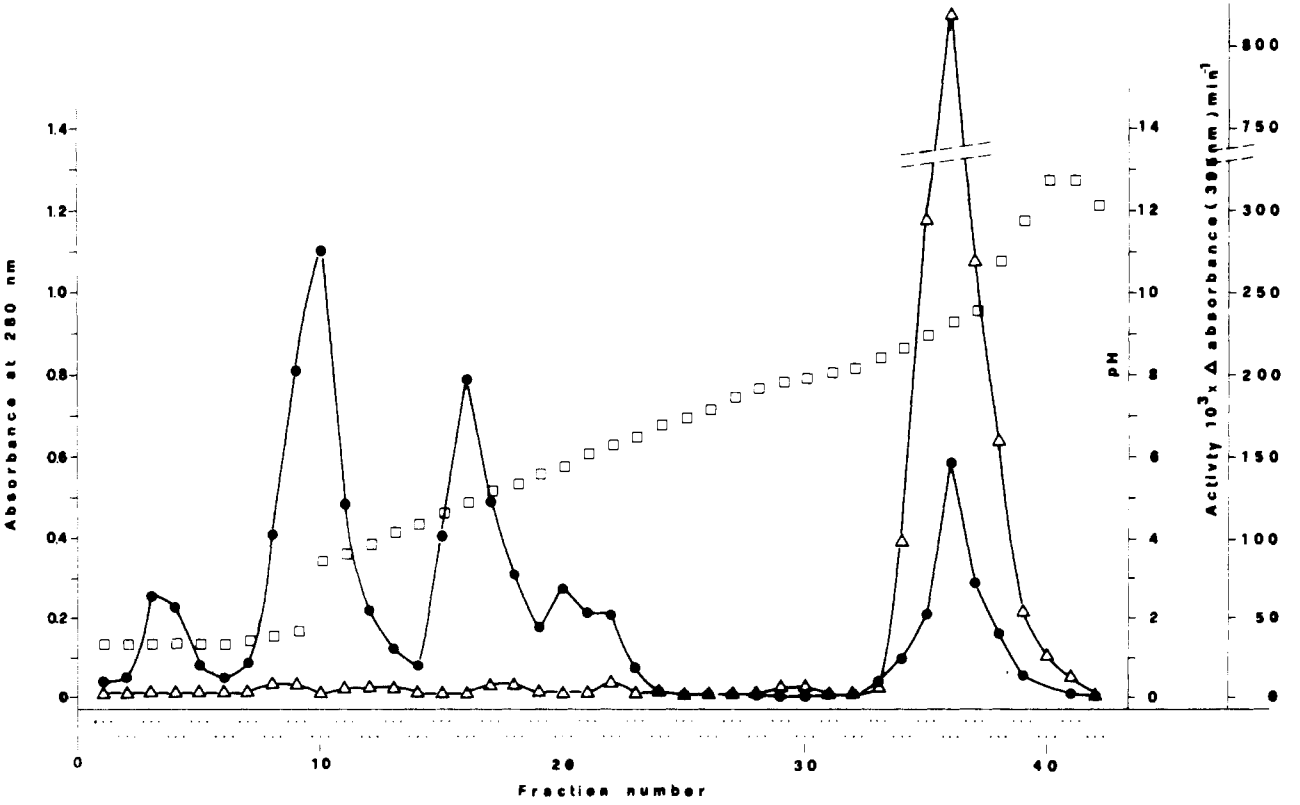

Figure 5. Isoelectric focusing of the fraction D_1 from ion-exchange chromatography: (△) *o*-diphenolase activity; (●) absorbance at 280 nm; (□) pH gradient.

chromatography (Figure 3) and isoelectric focusing (Figure 4), the *o*-diphenolase investigated shows itself to have multiple forms. It is difficult to make comparison between the two techniques since their separation principles are

different. The supernatant of the treatment with calcium phosphate gel (fraction C) shows three *o*-diphenolase isoenzymes both on DEAE-cellulose and isoelectric focusing columns. Their properties have been discussed above.

In one of our earlier studies (Interesse et al., 1981), isoenzymes of common wheat *o*-diphenolase were fractionated by using the same techniques and procedures. By chromatography and isoelectric focusing four isoenzymes were identified, whereas three have been detected in durum wheat. Furthermore, a comparison of the isoelectric points of the isoenzymes in the two varieties of wheat studied shows that the durum wheat sample lacks the isoenzyme focused at the lower isoelectric point, whereas the other three enzymatic forms are focused at the same p*I* (6.80), at a p*I* of 0.45 unit higher (pI = 5.45), and at a p*I* of 0.2 unit lower (p*I* = 9.40) than the corresponding fractions of common wheat.

An isoelectric focusing experiment was performed in order to check heterogeneity forms and/or a possible association of subnits of fraction D_1 recovered from ion-exchange chromatography. The attempt to separate the isoenzyme resulted in the generation of some maxima in the absorbance profile but only one (p*I* = 9.40) in that of the activity (Figure 5). This means that the enzyme associated with the fraction D_1 shows neither other multiple forms nor enzymatic subunits. Furthermore, since the enzyme associated with the fraction D_1 focuses at the same isoelectric point of fraction F_3 of Figure 4, we can clearly conclude that the two fractions active toward 4-methylcatechol are the same isoenzyme.

In conclusion, the study undertaken on the biochemical properties of a durum wheat sample *o*-diphenolase leads to a purification and characterization of the enzyme. The *o*-diphenolase isolated displays some properties (specific activity, pH profile, heterogeneity degree) that are different from those previously found in common wheat (Interesse et al., 1980, 1981). As it has also been shown by other authors (Feillet and Kobrehel, 1974; Lamkin et al., 1981; Kruger, 1976; Taneja et al., 1974; Tikoo et al., 1973), such differences may be useful in differentiating wheat types and/or classes.

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