Cathodic Peroxidases of Durum Wheat Flour

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ABSTRACT

Two cathodic peroxidases (C_1, C_2) were purified from durum wheat (*Triticum durum*) flour by ion-exchange chromatography. Both peroxidases appear to be homogeneous when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing. The isolated isoenzymes, both with a purity index ($A_{402}nm/A_{280}nm$) of 3.7, are polypeptides of 39.7 kDa (C_1) and 38.6 kDa (C_2), with pI >9.3. The calcium ion strongly activates the C_1 and C_2 isoenzymes, which increases their specific activity 92 and 690 times, respectively, when stored at pH 6.5. This effect is presumably due to the stabilization of the heme moiety structure, which was observed with the increase of the Soret band absorption. We consider this enzyme activity of great importance both in the production technology of high quality pasta and more generally in other bakery products.

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In the 1970s, peroxidase (EC 1.11.1.7) (POD) was purified from several sources and intensively investigated from different points of view. It is one of the most studied plant enzymes, given its physiological, genetic, analytical, and technological implications. This enzyme often has been used as a genetic marker and indicator of food quality; it was also the first enzyme to be used as a specific analytical reagent. For these reasons, POD is surely one of the milestones in the history of enzymology, although some aspects still remain unclear. There are, in fact, many open questions, mostly regarding its biological role, but also concerning its biosynthesis and chemical structure (van Huystee et al 1992). The best known POD, isoenzyme C from horseradish (HRP), consists of a heme prosthetic group and 308 amino acid residues, including four disulfide bridges in a single polypeptide chain of 33,890 Da, in which eight neutral carbohydrate side chains are inserted (Welinder 1979). This isoenzyme contains two Ca^{2+} ions that seem to have an important role both for maintaining the structural conformation of the protein and for its thermal stability. Conformational studies of HRP and other important POD isoenzymes indicated that the active sites are similar (Strickland et al 1968, Job and Dunford 1977), although there are some small differences due to the different amino acid and carbohydrate composition (Mazza et al 1968).

The activity of wheat POD was first reported by Bertrand and Muttermilch (1907). Durum wheat POD exists in multiple forms: up to 12 isoenzymes have been detected in immature whole seeds of some cultivars (Kruger and LaBerge 1974a). These isoenzymes were studied and utilized in physiology (Kruger and LaBerge 1974a, Peterson et al 1990) and as an aid to the identification of genotypes, and more generally, in phylogenetic studies (Kobrehel and Gauthier 1974, Kobrehel and Feillet 1975).

Although wheat POD is considered an important enzyme, especially for the quality and technological implications of the derived industrial food products, its molecular properties have been much less studied. There is little information on the properties of PODs in industrial wheat flour (Freimuth et al 1972, Jeanjean et al 1975) and even less on its biochemical characteristics, although some have been determined for PODs isolated from wheat germ (Tagawa et al 1959, Shin and Nakamura 1961) and immature and germinated seeds (Kruger and LaBerge 1974b).

To the best of our knowledge, the most recent report on PODs isolated from durum wheat Semolina was Jeanjean et al (1975).

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In addition, POD activity in wheat and flour has often been considered as one of the most important factors in producing the undesirable brown color of the derived products (Kobrehel et al 1972, Taha and Sàgi 1987). In this regard, it should be emphasized that an enzymatic activity has never been proven to be responsible for decreased yellow color in processed wheat products. Nevertheless, one indirect effect was described by Hsieh and McDonald (1984), who observed that a purified lipoxygenase (L-1) from durum wheat endosperm also showed POD activity. Thus, this enzyme seems to be crucial for color quality, as it effectively bleaches carotene. In fact, it seems to negatively affect the yellow color intensity of flour, presumably by decreased lutein content due to oxidation. This important finding is curious from the biochemical point of view. In fact, lipoxygenase and POD, while both being metalloenzymes with one iron atom per molecule, and having polypeptide chains with different structures and masses, show active sites containing the iron atom that are organized completely differently and are able to catalyze distinct reactions. It is well known that these reactions, while of the oxidative type, involve oxygen of diverse origins. Nevertheless, our preliminary study (S. Palmieri et al, *unpublished data*) indicated that different carotene bleaching activities occurred in the crude enzyme extracts of several durum wheat industrial semolinas. However, these bleaching activities were highly correlated with POD activity (McDonald 1979). This work suggested that, in durum wheat semolina, more than one enzyme was responsible for yellow color bleaching during the processing. Our preliminary work also indicated that lipoxygenase activities were very low, while those of polyphenol oxidase and especially POD was very high. This article describes the purification procedure and some physicochemical characteristics of two cathodic PODs in durum wheat flour.

MATERIALS AND METHODS

Materials

The semolina from durum wheat *(T. durum)* used in this study was supplied by Barilla SpA (Parma, Italy). Analyses of semolina (protein 13.1%, moisture 15.2%, and ash 0.88%) were performed in accordance with the AACC methods (1983).

Chemicals

CM-Sepharose F.F., diethylaminoethyl (DEAE)-Sephadex A-25, Mono S column, Superose 12 HR 10/30, PhastGel 12.5% PAA, isoelectric focusing (IEF) PhastGel 3-9, protein molecular weight standards for gel filtration and electrophoresis, and pI marker proteins were obtained from Pharmacia (Uppsala, Sweden). Bovine serum albumin (BSA) and Coomassie blue stain for the protein assays were obtained from Bio-Rad (Richmond, CA). The other reagents were of analytical grade.

Preparation of the Crude Extract

A sample of durum wheat semolina (300 g) was homogenized with 1.5 L of 0.05*M* sodium acetate buffer (pH 3.8) in an Ultra-Turrax homogenizer (model T45, Ika-Werk, Staufen, Germany) in an ice bath, initially for 5 min, and after standing 30 min, for an additional 5 min. The insoluble material was removed by centrifugation at 17,700 × *g* for 30 min at 4°C; the supernatant was adjusted to pH 7.5 by adding 1*M* NaOH. The precipitate was removed by centrifugation. The supernatant was 70% saturated with ammonium sulfate. After salting out at 4°C overnight, the insoluble proteins were recovered by centrifugation, dissolved, and extensively dialyzed against the starting eluting buffer of 0.05*M* sodium acetate (pH 5.4).

Purification

The crude active extract was applied in two separate runs to an HR 10/10 preparative column (Pharmacia) packed with CM-Sepharose F.F. and connected to a fast-protein liquid chromatograph (FPLC, Pharmacia). Proteins were eluted using a linear concentration gradient of 0.0–0.2*M* NaCl in the starting buffer, with a flow rate of 1.5 ml/min. Effluent was collected in 3.0-ml fractions. All the active fractions were collected and dialyzed against starting buffer to remove salt. In five consecutive runs, the equilibrated active solution was loaded on a Mono S HR 5/5 column (Pharmacia) equilibrated with the starting buffer at a flow rate of 0.7 ml/min. The chromatography was performed with a linear gradient of 0.0–0.2*M* NaCl in the starting buffer and monitoring the 2.1-ml eluent fractions at 280 nm with a Uvicord SII (Pharmacia).

The solutions containing isoenzymes C_1 and C_2 were separately dialyzed against 0.05*M* borate buffer (pH 10.2) and were loaded, in two separate runs, on to a 2.6- × 10-cm column packed with DEAE-Sephadex A-25. The active isoenzymes C_1 and C_2 were eluted in the void volume and completely recovered by washing the column. The active fractions were pooled and dialyzed against 0.05*M* acetate buffer (pH 4.5).

In two separate runs, each active isoenzyme was loaded on to a Mono S HR 5/5 column (Pharmacia) equilibrated with 0.05*M* acetate buffer (pH 4.5). Ion-exchange chromatography was performed using a linear gradient elution of 0.0–0.4*M* NaCl in 0.05*M* acetate buffer (pH 4.5) with a flow rate of 0.7 ml/min. The active fractions (1.4 ml) of pure C_1 and C_2 POD isoenzymes were pooled and stored at 4°C until used.

POD Assay

POD activity was determined by measuring the slope from the linear increase in absorbance at 470 nm due to oxidation of guaiacol to tetrahydroguaiacol in the presence of H_2O_2 (Chance and Maehly 1955). A typical assay mixture contained 2.20 ml of 40.3 m*M* guaiacol dissolved in 50 m*M* acetate buffer (pH 4.2); 0.05 ml of 2.0*M* Ca^{++}; and 0.25 ml of 29.4 m*M* H_2O_2. The assay mixture was loaded in a quartz cell with 10-mm path length; after adding a suitable aliquot of sample, the reactions were performed at 30°C in the temperature-controlled spectrophotometric cell compartment. One unit of POD activity was defined as a change of 1.0 absorbance unit per minute. The kinetic measurements were determined with a Kontron model 940 spectrophotometer.

Amino Acid Analysis

Homogeneous POD isoenzymes C_1 and C_2 were hydrolyzed by the gas-phase method in a vacuum at 105°C for 24 hr in 6*N* HCl and 1% (v/v) phenol. Amino acid analysis was performed after a precolumn derivatization procedure as described by Cohen et al (1993), using a Jasco (Japan Spectroscopy) high-performance liquid chromatograph equipped with a 980 PU and an 820-FP detector.

Protein Analysis

Protein concentrations were determined at all enzyme purification stages by the Coomassie blue dye binding method (Bradford 1976). BSA (1 mg/ml) was used as standard protein.

Electrophoresis Procedures

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) of isolated isoenzyme C_1 and C_2 were performed with a Phastsystem apparatus (Pharmacia, Sweden) using Phastgel 12.5% PAA and Phastgel IEF pH 3-9, respectively.

Spectrophotometry

To evaluate the effect of the Ca^{2+} ion on the absorbance of the Soret band, an aliquot of purified C_1 isoenzyme was diluted and dialyzed against 10 m*M* NaCl. After the spectrum was recorded, solid $CaCl_2$ was added directly to the cuvette to a concentration of 10 m*M*, then the spectrum was recorded again.

RESULTS AND DISCUSSION

Isolation of POD Isoenzymes

Table I shows the procedures employed and the results for POD C_1 and C_2 isoenzyme purification, which amounted to 300- and 261-fold enrichments with a specific activity of the 19.8 and 17.24 × 10^3 U/mg of soluble protein, respectively. In addition, at the end of purification procedure, the ratio of A_{402}nm/A_{280}nm

TABLE I
Purification of Peroxidase Isoenzymes C_1 and C_2 from Durum Wheat Flour

Purification Steps	Volume (ml)	Total Protein (mg)	Total Activity ($U \times 10^3$)	Specific Activity ($U\ mg^{-1} \times 10^3$)	Yield (%)	Purification (fold)	Purity Index (RZ)
Extract (pH 7.5)	1,380	1,242	189.06	0.152	99.3	2	ND[a]
70% $(NH_4)_2SO_4$ dialyzed	220	616	180.40	0.293	94.7	4	ND
CM-Sepharose (pH 5.4)	72	79.2	177.12	0.224	93.0	34	0.4
Mono S (pH 5.4)							
C_1	43	15.0	84.71	5.647	44.5	85	0.7
C_2	41	8.6	32.84	3.819	17.2	58	0.7
DEAE-Seph A-25 (pH 10.2)							
C_1	52	7.8	78.31	10.040	41.1	152	1.4
C_2	53	4.0	26.55	6.638	13.9	100	1.4
Mono S (pH 4.5)							
C_1	4.2	2.76	54.62	19.800	28.7	300	3.7
C_2	2.8	0.84	14.48	17.240	7.6	261	3.7

[a]Not determined.

(RZ value), which is used as an additional indicator of POD purity, was 3.7 for both isoenzymes. This value is about three times higher than that reported by Jeanjean et al (1975) for isoperoxidases A and B, isolated from durum wheat semolina. Furthermore, the specific activity values for C_1 and C_2 are the highest obtained for durum wheat POD to date, presumably due to the presence of calcium ion in our assay mixture, which is a strong activator of these PODs.

Preliminary extraction trials indicated that POD extracted with 0.05*M* acetate buffer (pH 3.8) gave the highest specific activity with the highest enzyme-unit yield. In addition, when the pH of the crude extract was increased to 7.5, more than half of the nonactive proteins were removed (Table I). Another important enrichment, with a minor loss of activity, was determined by the immobilization of the isoenzymes on CM-Sepharose at pH 5.4. This step, however, still does not allow the separation of the two isoenzymes. This separation is obtained by a second chromatography on a Mono S column at pH 5.4 (Fig. 1). This result was reached by trial and error in varying the chromatographic pH. This parameter was crucial for obtaining homogeneous POD isoenzymes that show similar molecular properties. In fact, as shown in Table II (and later in Fig. 5A,B), the two proteins have isoelectric points, molecular weight, and amino acid compositions that are similar but sufficiently different to permit their isolation. This result is also important from a practical point of view, as it permits the determination of the distribution of the POD activity in the two isoforms, which may prove to be interesting wheat-genotype markers. In our case, isoenzyme C_1 is about 70% of the total isolated activity.

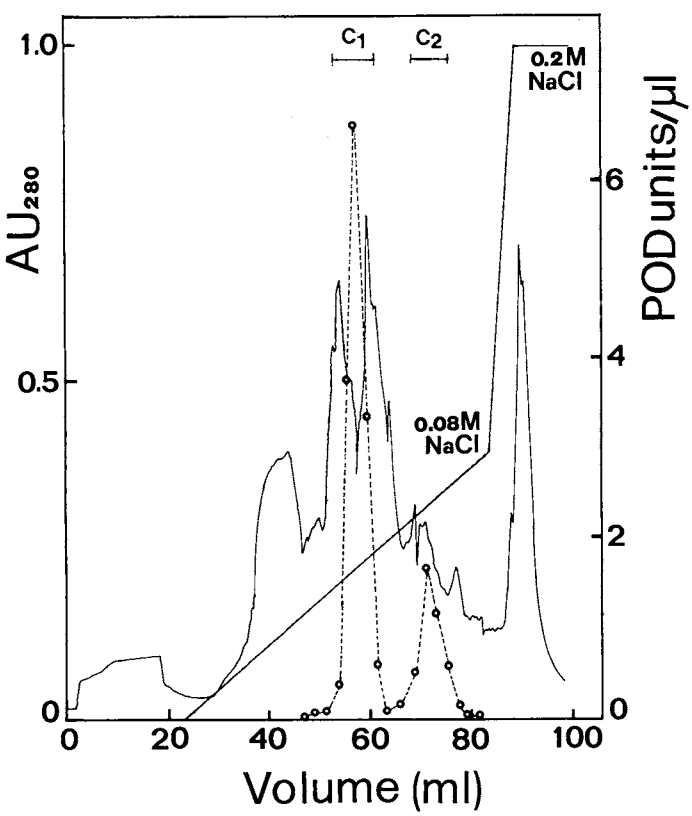

Fig. 1. Ion-exchange chromatography on the cationic exchanger column (Mono S HR 5/5) of the dialyzed pooled active fractions (15 ml) eluted from CM-Sepharose. Fractions were monitored at 280 nm (—) and assayed for peroxidase (POD) activity (o--o).

Another important purification step is the chromatography on DEAE-Sephadex A 25 (pH 10.2). Exploiting the high pI values of isoenzymes, C_1 and C_2 were eluted in the void volume of the column, whereas at least 50% of the contaminant proteins, which cannot otherwise be removed, remained bound to DEAE resin.

The final step of purification was again performed on a Mono S column, but in this case conditioned at pH 4.5. This lower pH value was chosen because it allows both POD isoenzymes to bind more strongly because of their basic pI. Figures 2 and 3 show that C_1 and C_2 isoenzymes elute after most of the nonactive proteins at the end of the chromatographic profiles.

Spectroscopy

Fig. 4A shows the absorption spectrum of C_1 isoenzyme in a 50 m*M* acetate buffer solution (pH 4.5) just eluted from the chromatography in the presence of ~0.25*M* NaCl. It is a typical POD spectrum, with a maximum at 399 nm due to the absorption of the heme, which constitutes the enzymes prosthetic group; a minimum at 300 nm; and a ratio of the 402/280 nm (RZ) absorbances of ~3.7. Absorption spectrum changes with varying pH and ionic strengths, especially at ~400 nm (data not shown).

TABLE II
Amino Acid Composition of Peroxidase Isoenzymes C_1 and C_2[a]

Residue	C_1	C_2
Asp	19.2	20.6
Thr	16.0	11.9
Ser	16.0	20.2
Glu	36.1	31.7
Pro	8.0	11.9
Gly	24.0	18.5
Ala	16.0	21.0
Val	24.0	18.9
Met	9.2	18.5
Ile	12.0	6.6
Leu	32.0	41.2
Tyr	24.0	18.5
Phe	20.0	16.5
Lys	14.0	17.3
His	4.0	3.3
Arg	8.0	7.4
Trp	2.4	2.5
Cys	3.2	4.1

[a]Values are nmoles of amino acid per nmole of protein.

These results reflect the ionization of the hydrophobic amino acids, which make up the environment surrounding the heme group, and its different orientation in the molecule. Also, a partial denaturation of the protein seems to occur, given that these changes are not completely reversible.

Fig. 4B shows the effect of the Ca^{2+} ion on the spectroscopic characteristics of C_1 after dialysis against 10 m*M* NaCl. As in the case of other PODs, and HRP in particular (Haschke and Friedhoff 1978), C_1 is positively affected by the presence of 10 m*M* Ca^{2+}, which presumably allows the protein to maintain its optimal structural conformation. This effect is also confirmed by the kinetic data for both C_1 and C_2 (data not shown). In addition, it is easy to note that C_1, after dialysis (presumably without Ca^{2+}), shows both an RZ and an activity lower (~25%) than the same isoenzyme just eluted from the column (Fig. 4A).

Molecular Mass and pI Determinations

The purified POD isoenzymes C_1 and C_2 migrated in SDS-PAGE gel as single polypeptides of 39.7 and 38.6 kDa, respectively (Fig. 5A). The molecular mass was also determined by gel-filtration chromatography on a Superose 12 column equilibrated with 50 m*M* phosphate buffer (pH 7.0) containing 0.15*M* of NaCl and connected to FPLC apparatus (Pharmacia). The column was calibrated with standard proteins, BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) with no detergents in the chromatographic solution. Molecular mass determinations gave values about 30% lower than those estimated by SDS-PAGE. The explanation for this lies in the interaction between our high basic C_1 and C_2 proteins and the column media that occurs in our experimental conditions. This phenomenon was also observed in a previous work on highly basic proteinase inhibitors isolated in oil-bearing cruciferous seeds (Visentin et al 1992).

The pI of C_1 and C_2 were both around 9.3, which may be slightly higher than the pI of the most basic standard protein (Fig. 5B). These values cannot be checked precisely because of limitations in commercially available ampholine-PAG plates. Nevertheless, this experiment confirms that C_1 and C_2 are homogeneous polypeptides that migrate toward the cathode as single bands. In fact, the lanes corresponding to the PODs do not present other extraneous proteins.

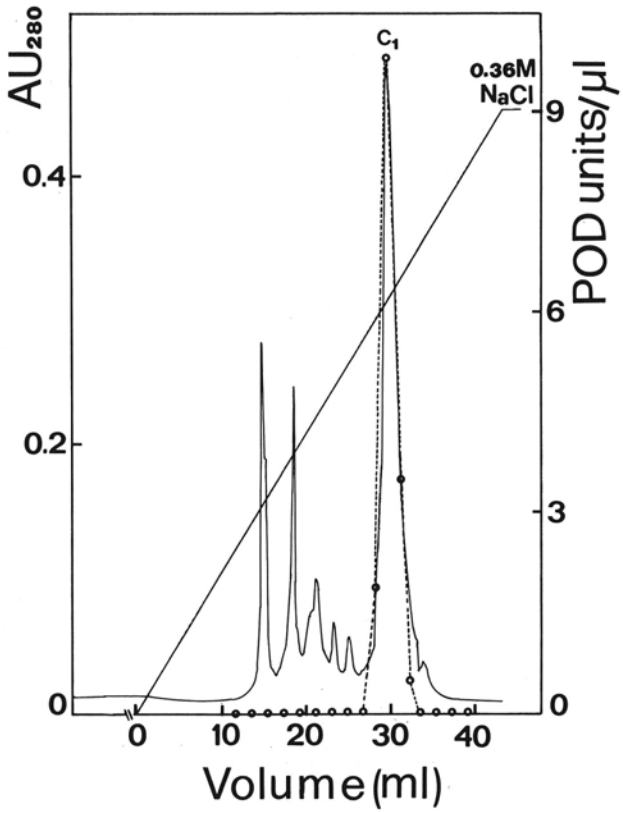

Fig. 2. Ion-exchange chromatography on the cationic exchanger column (Mono S HR 5/5) of the dialyzed pooled active fraction C_1 eluted in the void volume of a diethylaminoethyl-Sephadex A-25 conditioned at pH 4.5. The 1.4-ml fractions were monitored at 280 nm (—) and assayed for peroxidase (POD) activity (o--o).

Amino Acid Compositions

The amino acid compositions of C_1 and C_2 PODs are shown in Table II, although these are approximate compositions, given that tryptophan and cystein were not directly determined but only estimated. It is interesting that although C_1 and C_2 are basic proteins, they are not particularly rich in lysine and arginine. The compositions of the two isoenzymes are similar but differ significantly in methionin and isoleucin content. This has no effect on the prevalence of hydrophobic residues in these isoenzymes, however.

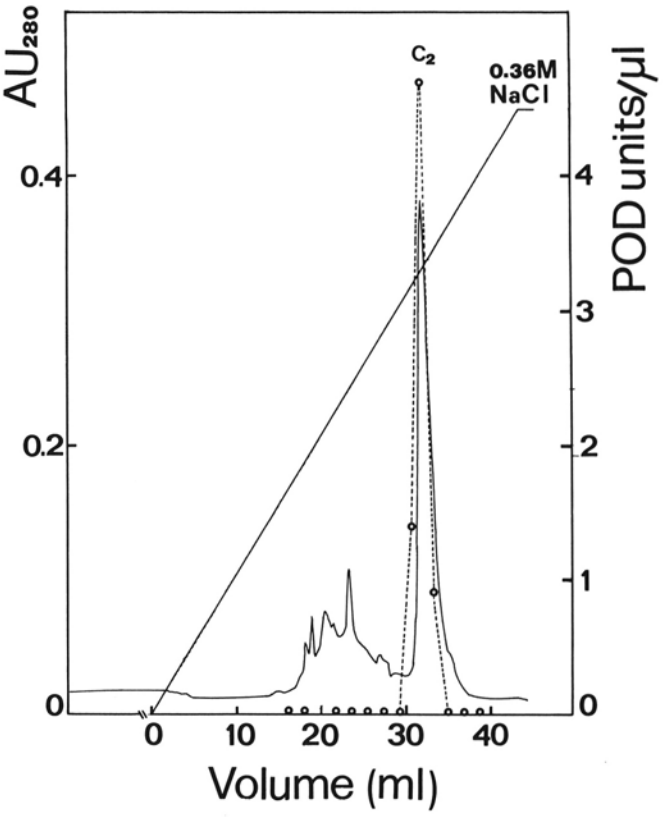

Fig. 3. Ion-exchange chromatography of the active fraction C_2. Chromatographic conditions were the same as in Fig. 2. POD = peroxidase.

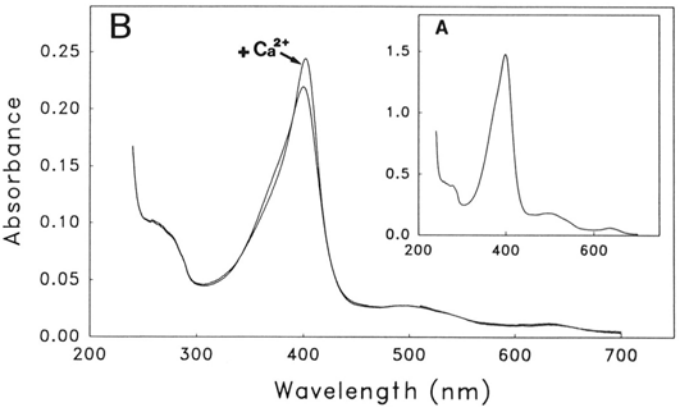

Fig. 4. A, Spectrum of peroxidase isoenzyme C_1 eluted from the Mono S column. **B,** Effect of 10 m*M* Ca^{2+} ion on the spectral properties of the heme, contained in peroxidase isoenzyme C_1.

Calcium Ion

Jeanjean et al (1975) reported the presence of a POD inhibitor in durum wheat semolina that they removed with DEAE ion-exchange chromatography at pH 10.2. This finding was presumably accepted, without further considerations, solely on the basis of the increase of total POD activity observed in the collected fractions. In our case, this result was not confirmed, even when the conditioned crude extract was directly applied to a DEAE column, as in the cited report. In addition, to demonstrate the presence of a POD inhibitor in the crude extract of durum wheat semolina, the retained proteins on the DEAE column were totally recovered after the POD elution in the void volume using 1*M* NaCl. After dialysis, this solution was used for the POD inhibition assay. Even in this case, no significant inhibition of POD activity was detected. Therefore, it is reasonable to assume that the increased total activity reported by Jeanjean et al (1975) was due to the absence of calcium ions in the assay mixture they used. In fact, on the basis of several experiments designed to demonstrate the importance of this cation for POD activity, we strongly suggest that the presence of at least 10 m*M* Ca^{2+} ions in the POD assay mixture is crucial for obtaining linear enzymatic kinetics and precise, reproducible activity data. In this regard, Figure 6 shows the influence of the Ca^{2+} ion concentration on POD isoenzymes C_1 and C_2. It is clear that, as mentioned above, this cation is extremely important for maintaining the optimal structure in the heme vicinity, which also corresponds to the maximum catalytic activity of the enzyme (van Huystee et al 1992). When the Ca^{2+} ion was presumably removed by extensive dialysis against buffer at pH 6.5, we observed a minimal activity both for C_1 and C_2 (0.21 and 0.025, respectively). Although the isoenzymes were differently activated (C_1 appears to be less sensitive to Ca^{2+}), both displayed a maximum increase of activity up to 10 m*M*, then reaching a plateau at ~20 m*M*. These trends for POD activity are rather different from those reported by JeanJean et al (1975), who indicate, for instance, a maximum activation at ~50 m*M*. Furthermore, with our PODs, we did not observe any significant inactivation at higher Ca^{2+} concentrations (data not shown). These different results presumably depend on the genotype considered, and the purity of the proteins, or both. Note also that in Figure 7, the different behaviors of the two isoenzymes in the presence of 20 m*M* Ca^{2+} ions and varying pH. In fact, the first effect is that C_1 remains more active than C_2 in the 4.2–6.0 range, the second is that C_1 with Ca^{2+} ions shifts the optimal pH from ~4.2 to 4.8. This is further evidence that Ca^{2+} ions produce a favorable change in conformation of the prosthetic heme group in the enzyme.

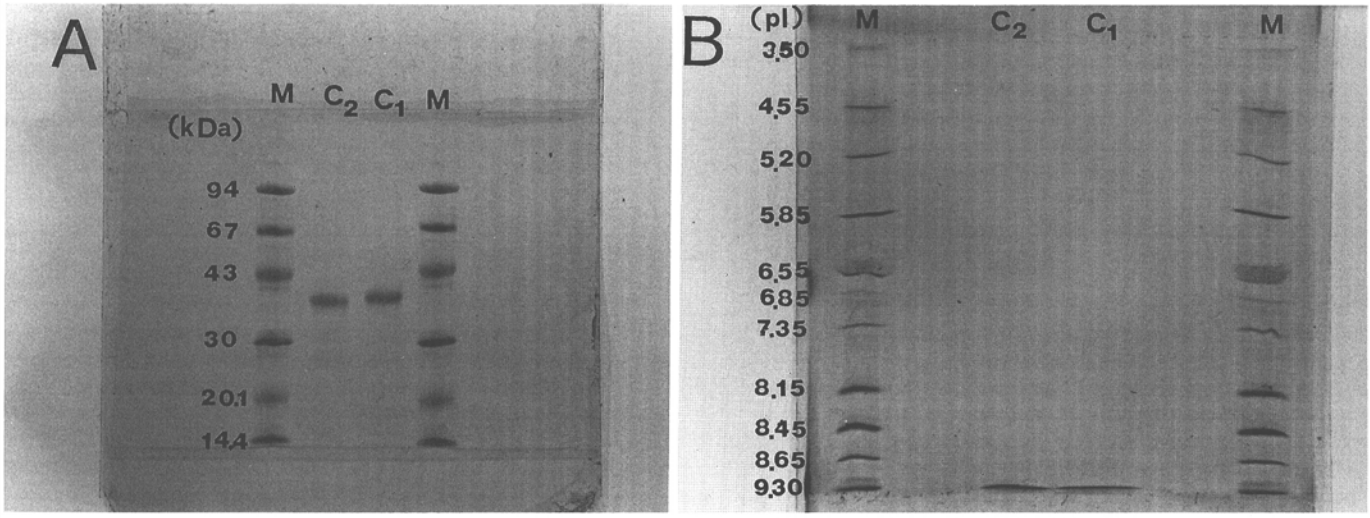

Fig. 5. A, Electrophoresis of peroxidase isoenzymes C_1 and C_2. M = low molecular weight standards (Pharmacia). **B,** Isoelectric focusing of peroxidase isoenzymes C_1 and C_2. M = protein standards with pI range 3.5–9.3.

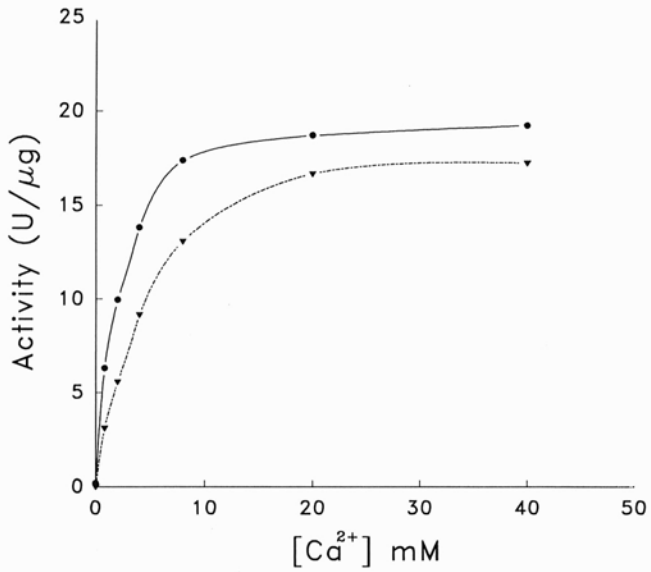

Fig. 6. Peroxidase activity of isoenzymes C_1 (●) and C_2 (▲) at pH 4.2, previously conditioned at pH 6.5 at different Ca^{2+} concentrations.

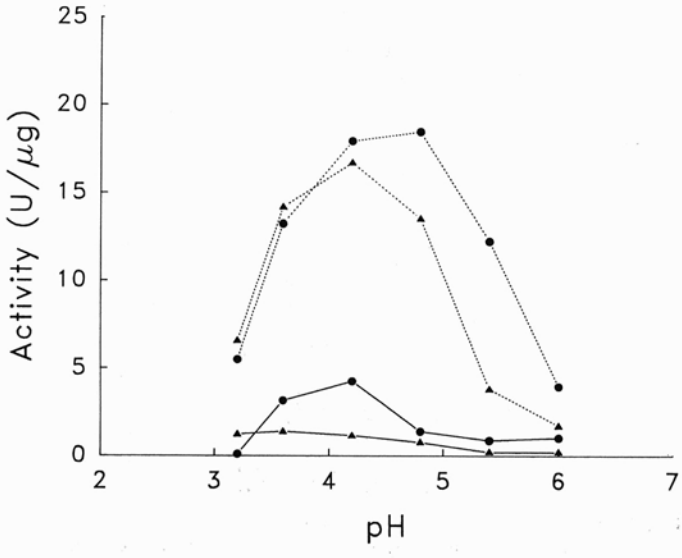

Fig. 7. Peroxidase activity of isoenzyme C_1 (●) and C_2 (▲) at different pHs with (· · ·) and without (—) 20 m*M* Ca^{2+}. The isoenzymes were previously conditioned at pH 4.0.

TABLE III
Effect of pH and Calcium on the Isoenzymatic Peroxidase (POD) Activity

	Pod Activity (U/μg)			
	pH 6.5		pH 4.0	
Isoenzyme	−Ca	+Ca[a]	−Ca	+Ca[a]
C_1	0.21	19.24	4.32	19.09
C_2	0.025	17.26	1.13	17.21

[a]40 m*M*.

Effect of pH

The optimum pH for the two isoenzymes in the presence of Ca^{2+} ions is significantly different; without this cation, it is difficult to evaluate, given the very low activity determined for C_2 (Fig. 7). Although POD activity is remarkably affected by the pH of the reaction mixture, it is important to emphasize that the conditioning pH of the enzyme is also crucial for determining the catalytic peroxidation reaction rate without Ca^{2+}. In this regard, Table III shows POD activity data determined at pH 4.2 (the typical pH of the reaction mixture) but with the isoenzymes conditioned at pH 6.5 and 4.0. At pH 4, without Ca^{2+} ions, the activity of C_1 and C_2 are ~21 and 45 times higher than at pH 6.5. However, this striking difference is completely cancelled when 40 m*M* Ca^{2+} is added. This finding is also confirmed when the Ca^{2+} concentration reaches 10 m*M*, although at lower concentrations the difference in activity is very small (data not shown). In addition, Table III shows that, while at pH 6.5, the presence of Ca^{2+} increases the activity of C_1 and C_2 92 and 690 times, respectively; at pH 4.0, they were activated only ~4 and 15 times, respectively. This indicates without doubt that acidic pH stabilizes the enzyme, producing an effect similar to calcium, but also prevents enzyme inactivation, which easily occurs by heme detachment at higher pH.

CONCLUSIONS

The high purity achieved in the isolation of the two semolina POD isoenzymes (3.7 RZ) permitted a series of studies that were previously impossible or difficult to interpret. It is, in fact the first time that these isoenzymes have been studied in a homogeneous form, as confirmed by SDS-PAGE and IEF.

These studies emphasize the importance of the calcium ion and the pH to correctly study this activity, which has been of great importance in genetic improvement and in improving the quality of products made from durum wheat. Our results indicate that the pH of both the reaction and of the enzyme conditioning, as well as the presence and concentration of Ca^{2+} ions, greatly affect semolina POD activity. In our opinion, these findings are of considerable theoretical and practical importance: first, for determining the different molecular characteristics of the two isoenzymes; and second, to improve technology for obtaining high-quality pasta and other products from durum wheat.

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