Purification and Some Properties of Catalase from Wheat Germ (*Triticum aestivum* L.)

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Two isoforms of catalase, CAT-1 and CAT-2, were purified from wheat germ after extraction, ammonium sulfate precipitations, hydrophobic chromatography, and two ionic-exchange chromatographies. The global yields and the purification factors were close to 3% and 50 for CAT-1 and close to 6% and 100 for CAT-2. Both isoforms exhibit an optimum activity at pH 7. When pH was decreased from 7 to 5.6, CAT-1 showed a decreasing affinity for its substrate, whereas the opposite was found for CAT-2. Both isoforms were irreversibly denaturated when exposed to acidic pH, CAT-1 being more sensitive than CAT-2. Conversely, CAT-2 appeared to be more sensitive to inhibitors. The rate as well as the extent of denaturation during incubation with 3-amino-1,2,4-triazole (AT) were higher with CAT-2 than with CAT-1. Guaiacol is a competitive inhibitor more potent with respect to CAT-2. The difference in affinity for hydrogen peroxide as well as the poor stability of CAT-1 in acidic medium suggests that this isoform could be less effective during dough mixing.

Keywords: *Catalase; wheat germ; purification; properties*

INTRODUCTION

During dough mixing, many oxidative reactions take place. Some of them are catalyzed by oxidoreducing enzymes, which are of interest for baking industries because they are of importance for the dough rheological properties and for the organoleptical properties of bread (Grosch, 1986). Among the different oxidoreductases present in wheat, lipoxygenase (LOX: EC 1.13.11.12) is one of the most studied due to its well-known effect during dough mixing (Nicolas and Potus, 1994). This enzyme catalyzes the oxidation of polyunsaturated fatty acids in the free and monoglyceride forms by molecular oxygen (Tait and Galliard, 1988; Castello et al., 1998). By coupled oxidation reactions, lipoxygenase is able to degrade carotenoid pigments (Nicolas, 1978) and to promote a loss of flour sulfhydryl groups (Graveland et al., 1978; Shiiba et al., 1991). This results on the one hand in dough bleaching and on the other hand in an increase of mixing tolerance and relaxation time of dough, causing an enhancement of the loaf volume (Frazier et al., 1977; Hoseney et al., 1980; Cumbee et al., 1997). Moreover, lipoxygenase-mediated oxidation of polyunsaturated fatty acids also generates volatiles during proofing and baking, such as hexanal and hexanol, which induce a modification of bread aroma (Drapron et al., 1974; Addo et al., 1993).

Ascorbic oxidase (EC 1.10.3.3) and glutathione-dehydroascorbate oxidoreductase (EC 1.8.5.1) have been also largely studied (Every et al., 1996; Kaid et al., 1997). However, their effects on dough rheology are obviously limited to the wheat flours supplemented with ascorbic acid.

Peroxidase (POD: EC 1.11.1.7) and catalase (CAT: EC 1.11.1.6) are present in high amounts in wheat flour (Honold and Stahmann, 1968; Kruger, 1977; Kieffer et al., 1982). However, few studies have been devoted to their effects in breadmaking (Hawthorn and Todd, 1955; Nicolas, 1978; Kieffer et al., 1981; Gelinas et al., 1998). On the basis of studies in model solutions, it has been proposed that POD can promote in the dough the oxidative gelation of pentosans (Geismann and Neukom, 1973; Izydorczyk et al., 1990; Figueroa-Espinoza and Rouau, 1998) and/or the polymerization of proteins (Matheis and Whitaker, 1984). Moreover, the baking performance of wheat flour can be improved by addition of POD from different sources (Kieffer et al., 1981; Van Oort et al., 1997).

The mechanism remains unclear probably because of the questionnable formation of hydrogen peroxide, their primary substrate, during dough mixing. However, recently, Liao et al. (1998) have indicated that, during fermentation, yeast produced hydrogen peroxide, which had an oxidant effect on the dough rheology. This hydrogen peroxide production could then explain the improving effect by the activation of POD. A similar mechanism could explain the improving effect of the addition of glucose oxidase (or hexose oxidase) to wheat flour (Van Dam and Hille, 1992; Martinez-Anaya and Jimenez, 1998; Wikström and Eliasson, 1998; Poulsen and Bak Hostrup, 1998; Vemulapalli et al., 1998). The latter enzyme oxidizes glucose (or a number of mono- and oligosaccharides in the case of hexose oxidase) in the presence of oxygen and produces hydrogen peroxide.

Whereas several works have been devoted to the wheat POD, including the purification of different isoenzymes (Jeanjean et al., 1975; Zmrhal and Machackova, 1978; Iori et al., 1995; Billaud et al., 1999), surprisingly enough, very few studies have been published on the physicochemical and kinetic properties of the wheat CAT (Irvine et al., 1954), and to our knowledge, this enzyme has never been purified.

Both peroxidases and catalases have hydrogen peroxide as substrate. The knowledge of their kinetic

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properties is needed to understand how these catalysts compete during mixing of the doughs supplemented with hydrogen peroxide producing enzymes. It is the purpose of this work to purify the wheat CAT in order to determine some of their properties. Catalases have been isolated from numerous procaryotes and eucaryotes (Chandlee et al., 1983; Chan et al., 1978; Esaka and Asahi, 1982; Kato et al., 1997; Kikuchi-Torii et al., 1982; Nadler et al., 1986). They are usually composed of four identical subunits, and their molecular weights are found to be close to 240 kDa (Robinson, 1991). Among the different milling fractions, wheat germ is by far the richest one in CAT activity (Hawthorn and Todd, 1955); accordingly, the purification has been carried out from this fraction. In further studies, we plan to study the interactions among wheat oxidoreducing enzymes. Therefore, one of our objectives was to obtain a CAT extract free from LOX and POD activities. Consequently, the LOX and POD activities were assayed in all the fractions obtained during the purification procedure.

MATERIALS AND METHODS

Plant Material. Industrial wheat germ was provided by les Moulins Soufflet (Nogent-sur-Seine, France). It was defatted by cold acetone according to Nicolas et al. (1982).

Chemicals. Diethylaminoethyl (DEAE)-Sepharose CL 6B, polyacrylamide gradient gels 10−15% for electrophoresis in native conditions were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Bovine serumalbumin used as standard protein was obtained from Sigma (St. Louis, MO). The ampholytes solution of Biolytes pI 3−9 and Coomassie Blue were purchased from Bio-Rad (Ivry-sur-Seine, France). Toyopearl-Butyl 650M (TSK) gel was obtained from Merck (Nogent-sur-Marne, France).

Extraction and Purification Procedure. Unless otherwise stated, all steps were performed at 0−4 °C. Six grams of defatted germ was homogenized with 60 mL of sodium phosphate buffer (0.1 M, pH 8.0) using an Ultra Turrax homogenizer (IKA, Stauffen, Germany) for 15 s followed by a 30 s rest period and another 15 s treatment. Homogenate was immediately centrifuged (30000*g* for 30 min), and the supernatant constituted the crude extract. Powdered ammonium sulfate (AS: 106 $g.L^{-1}$, 20% sat) was slowly added to the crude extract, and the sample was kept for 3 h. After centrifugation (10000*g* for 20 min), 143 $g{\cdot}L^{-1}$ of AS (45% saturated) was added to the supernatant S_{20}. After one night at 4 °C, the extract was centrifuged (10000*g* for 20 min). The active pellet (C_{45}) was resuspended in 60 mL of sodium phosphate buffer (0.1 M, pH 7.5) containing 0.3 M AS (buffer A) and dialyzed overnight against the buffer A. After centrifugation (20000*g* for 20 min), the dialyzed extract (C_{45d}) was loaded onto a 30- x 1.6-cm column of TSK (60 mL bed volume−hydrophobic chromatography) previously equilibrated with the buffer A at a flow rate of 80 $mL{\cdot}h^{-1}$. After elution of the unbound proteins by two bed volumes of the buffer A, the proteins still bound were washed successively with buffer A without AS (two bed volumes), distilled water (two bed volumes), and ethylene glycol-water (50/50; V/V) (two bed volumes). Each 5 mL fraction was estimated for CAT, POD, and LOX activities, and UV absorbance was measured at 280 nm. CAT-active fractions from the hydrophobic chromatography were pooled and dialyzed overnight against a phosphate buffer (50 mM, pH 8.0; buffer B). The dialyzed extract was loaded onto a 20- × 1.6-cm column of DEAE-Sepharose column (40 mL bed volume, anion-exchange chromatography) equilibrated with the buffer B at a flow rate of 90 $mL{\cdot}h^{-1}$. After elution by two bed volumes of buffer B of unbound proteins (containing CAT-1), the major part of the CAT activity (CAT-2) was eluted by a linear gradient of AS (0−0.6 M) in five bed volumes of buffer B. The proteins still bound were eluted by two bed volumes of buffer B containing 1 M AS. Each 5 mL fraction was estimated for CAT and POD, and UV absorbance was measured at 280 nm. The fractions containing the CAT-1 activity were pooled and dialyzed overnight against sodium phosphate buffer (10 mM, pH 7.0; buffer C). The dialyzed extract was loaded onto a 20- × 1.6-cm column of DEAE-Sepharose CL6B (40 mL bed volume) equilibrated with buffer C at a flow rate of 70 $mL{\cdot}h^{-1}$. After elution of unbound proteins (containing the POD activity) by two bed volumes of buffer C, the CAT-1 activity was eluted by a linear gradient of AS (0−0.6 M) in five bed volumes of buffer C. Each 5 mL fraction was estimated for CAT and POD activities, and UV absorbance was measured at 280 nm. Active fractions of CAT-1 and CAT-2 were pooled and stored at −20 °C without loss of activity for at least 3 months.

Electrophoresis Experiments. Electrophoresis experiments were performed with the Phastsystem (Pharmacia) using Phastgels 10−15% (electrophoresis in native conditions). The migration conditions and the $AgNO_3$ staining for protein bands were carried out according to the manufacturer instructions (Phastsystem booklet). CAT activity was detected on gels according to Woodbury et al. (1971). Activity staining was obtained after a 20 min incubation in 0.003% hydrogen peroxide followed by an incubation in 0.2% ferric chloride and potassium ferricyanide. Catalase appeared as yellow bands in a green background.

Catalase Assay. In routine measurements, the catalase activity was determined polarographically at 30 °C, by following the O_2 production with a Clark-type oxygen electrode in a 100 mM sodium phosphate buffer solution at pH 7 saturated by air and containing hydrogen peroxide (1 M). When pH was varied, the same phosphate buffer (0.1 M) was used between pH 5.7 and 8, and sodium acetate buffer (0.1 M) was used between pH 4 and 5.6. For kinetic studies and inhibition by guaiacol (4.5, 10, and 20 mM), the hydrogen peroxide concentration varied between 40 mM and 1 M. Kinetic parameters were determined by using nonlinear regression data analysis software (Leatherbarrow, 1987). For denaturation by 3-amino-1,3,4-triazole (AT) between 0.15 and 50 mM, the routine assay conditions were used without elimination of AT. All assays were performed in duplicate, and activity is expressed in μkat (μmol of oxygen formed per second in the assay conditions).

Peroxidase Assay. The POD activity was measured by a spectrophotometric method based on the increase in absorbance at 470 nm and 30 °C as described by Iori et al. (1995) with minor modifications (Billaud et al., 1999). The reaction mixture contained guaiacol (20 mM), H_2O_2 (8 mM), and $CaCl_2$ (20 mM) in sodium acetate buffer (0.1 M, pH 5.3). The activity was determined by the maximal slope from the linear increase in absorbance at 470 nm. One unit of activity is defined as a change of one absorbance unit per second for a total volume of 3 mL in the assay conditions.

Lipoxygenase Assay. The LOX activity was determined polarographically using linoleic acid (5 mM) dispersed in a sodium phosphate buffer solution (0.1 M, pH 6.5) containing Tween 20 (0.125%) and saturated by air at 30 °C as described by Nicolas et al. (1982). Activity is expressed in nkat (nmol of oxygen consumed per second in the assay conditions).

Protein Assay. Protein content of the different extracts was determined by the dye-binding method of Bradford (1976) using bovine serumalbumin as a standard.

Determination of pI. Isoelectric focusing (IEF) in liquid-phase medium was performed using a Rotofor system (Biorad). The C_{45d} extract of catalase was dialyzed overnight at 4 °C against water supplemented with 1% glycine and then diluted to ∼50 mL with water. Ampholyte (pH 3/10) solution and glycerol were added to a final concentrations of 2 and 10%, respectively. The solution was loaded onto the Rotorfor cell without further treatment. NaOH (0.1 M) and H_3PO_4 (0.1 M) were used as anode and cathode electrode solutions. Focusing was done at 12 W constant power for 4 h at 4 °C. Recorded voltages varied between 500 and 1400 V. Catalase activity (routine assay conditions), pH value, and absorbance value at 280 nm were measured on each fraction of the 20 fractions collected.

Table 1. Purification of Catalase from Wheat Germ (6 g)

			catalase				lipoxygenase	peroxidase
step[a]	total volume (mL)	total protein (mg)	total activity (μkat)	specific activity (μkat/mg)	yield (%)	purification factor	total activity (nkat)	total activity (UA/s)
crude extract	60	708	1080	1.53	100		9000	870
S_{20}	61	520	1068	2.05	99	1.3	8730	853
C_{45d}	49	175	713	4.07	66	2.7	7350	148
TSK	50	2.75	371	135	34	88	610	10
TSK_d	52	2.47	234	95	22	62	~0	10
$DEAE_1$ CAT-1	67	1.16	59	51	5.5	33	0	2
CAT-2	20	0.43	63	147	5.8	96	0	0
$DEAE_{1d}$ CAT-1	72	1.1	39.6	36	4.3	24	0	2
$DEAE_2$ CAT-1	25	0.42	35	83	3.2	54	0	0

[a] S_{20} = supernatant, C_{45d} = dialyzed solution, TSK = TSK 650M butyl, TSK_d = TSK dialyzed solution, $DEAE_1$ = diethylaminoethyl Sepharose CL6B (first chromatography), $DEAE_{1d}$ = $DEAE_1$ dialyzed solution, $DEAE_2$ = diethylaminoethyl Sepharose CL6B (second chromatography).

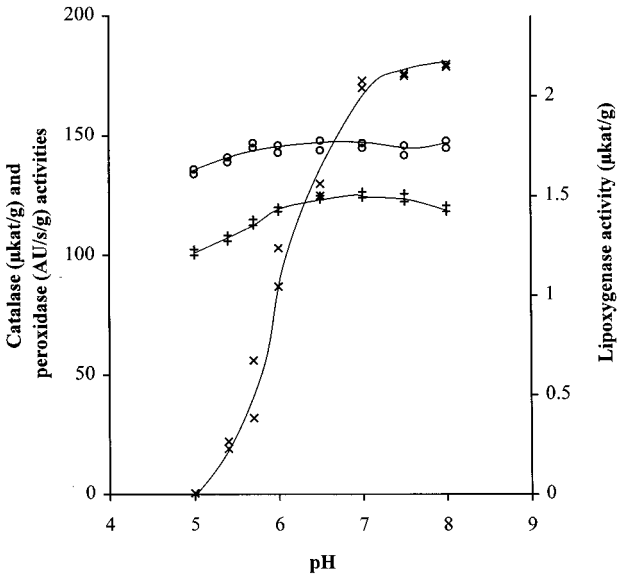

Figure 1. Effect of the pH of extracting buffer solution (acetate pH 5−5.6, phosphate pH 5.7−8, and Tris/HCl 7.2−8.6) on the catalase (—X—), peroxidase (—○—) and lipoxygenase (—+—) of wheat germ.

RESULTS AND DISCUSSION

Preliminary experiments were conducted with different buffers solutions between pH 5 and 8. The CAT extracted from defatted germ is highly sensitive to the pH of the extracting buffer solution (Figure 1). Thus, the CAT activity of the extract is almost 0 for pH = 5; it increases steadily between pH 5 and 7, then slightly between pH 7 and 8. Conversely, the LOX activity of the extract is hardly affected by the pH buffer. It is maximum at pH 7.5, and a loss close to 20% is found at pH 5. Under the same conditions, the POD activity of the extract is not affected by the pH between 5 and 8. These results are close to those obtained by Delcros et al. (1998), who studied the influence of pH on the extractable activities of the same enzymes from wheat flour and wheat dough. Consequently, a 0.1 M phosphate buffer at pH 8.0 was used to obtain the CAT crude extract from defatted wheat germ.

Purification Procedure. Typical results of the purification procedure of CAT from 6 g of defatted wheat germ are given in Table 1. Values concerning the LOX and POD activities of the different fractions are also included in Table 1 to follow their elimination during the purification of CAT.

After extraction, the AS treatments yielded an almost 3-fold purification with a yield of 66%. In these preliminary steps, 75% of proteins were eliminated as well as close to 20% of the LOX activity and more than 80% of the POD activity. For these two enzymes, the bulk of the eliminated activity was in the S_{45} supernatant. The latter result is in agreement with the findings of Billaud et al. (1999), who indicated that higher concentrations of AS are needed to precipitate the wheat germ peroxidases. These authors used a 20−70% AS cut in their purification procedure of wheat germ POD.

The hydrophobic chromatography on TSK butyl 650M allowed a close to 30-fold purification of the CAT, which was concentrated in a single peak eluted with the phosphate buffer (0.1 M at pH 7.5) without AS (Figure 2B). The bulk of the LOX and POD activities present in the C_{45d} fraction were eliminated with the unbound proteins for the POD activity and with the proteins eluted with water for the LOX activity (Figure 2A). Thus, although the yield of this specific step was only slightly higher than 50%, it allowed an almost total elimination of the contaminating activities of LOX and POD.

Two ionic-exchange chromatographies using DEAE Sepharose CL6B are needed for further purification of the wheat germ CAT. In the first step, two isoforms of CAT were separated, namely CAT-1 with the unbound proteins and CAT-2 eluted by increasing the buffer ionic strength (Figure 3). The latter CAT isoform, CAT-2, was obtained with an overall purification factor close to 100 and a global yield close to 6% and was free of POD activity. A second ionic- exchange chromatography applied to the CAT-1 fraction allowed its concentration (purification factor close to 50 and global yield slightly higher than 3%) and the total elimination of the residual POD activity (Figure 4). In this purification procedure, the highest losses occurred during the chromatographic steps and the dialysis steps between two chromatographies emphasizing the weak stability of the purified CAT from wheat germ in buffer solutions of low ionic strength.

Some Physicochemical Properties of CAT-1 and CAT-2. *Determination of Isoelectric Points.* The isoelectric points of CAT-1 and CAT-2 were determined by IEF in liquid-phase medium using a pH gradient from 3.8 to 9.3. The elution profile obtained from a C_{45d} extract shows a broader peak of CAT activity between pH 5.5 and 7 with a maximum at pH 6.3 and a shoulder at pH 5.8 (Figure 5). These pH values probably correspond to the isoelectric points of CAT-1 and CAT-2, respectively, and are in agreement with the isoelectric points of 5.5 and 6, which have been found for catalases purified from beef liver (Schonbaum and Chance, 1976) and tobacco

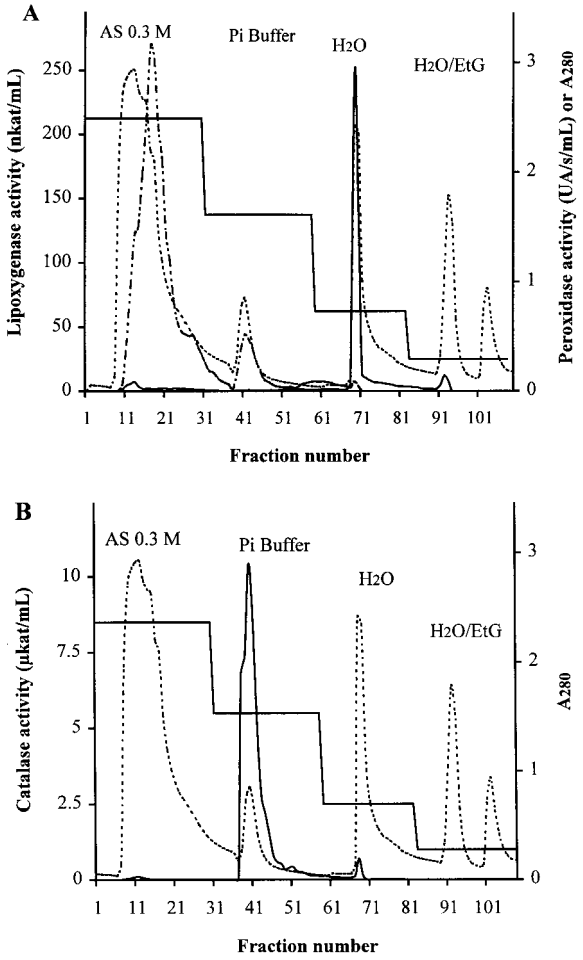

Figure 2. Hydrophobic chromatography on TSK butyl 650M of C_{45d} extract. A: Absorbance at 280 nm (- - -), peroxidase activity (•-•-•), and lipoxygenase activity (—) B: Absorbance at 280 nm (- - -) and catalase activity (—). For chromatographic conditions see Materials and Methods. π buffer = sodium phosphate buffer (0.1 M, pH 7.5). EtG/H_2O = ethylene glycol/H_2O (50/50; v/v).

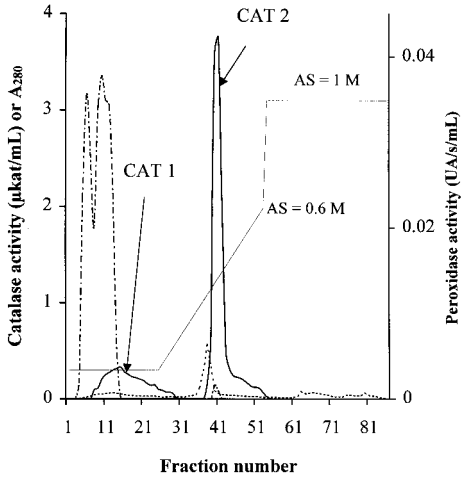

Figure 3. Anion-exchange chromatography on diethylaminoethyl (DEAE) Sepharose CL6B of TSK extract. Absorbance at 280 nm (- - -), peroxidase activity (•-•-•), and catalase activity (—).

(Havir et al., 1996). However, the total amount of CAT activity in the IEF fractions represented less 10% of the initial activity of the C_{45d} extract. This very low yield does not allow us to determine the relative proportions of CAT-1 and CAT-2 in the C_{45d} extract. IEF carried out

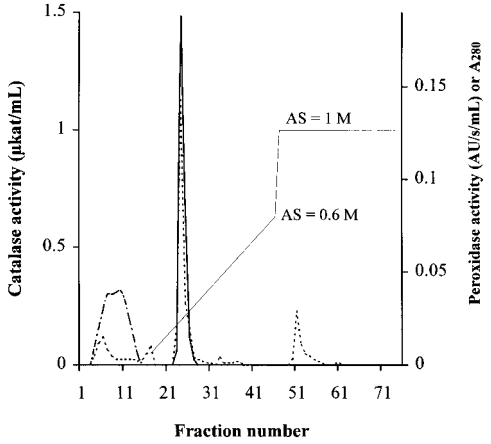

Figure 4. Anion-exchange chromatography on diethylaminoethyl (DEAE) Sepharose CL6B of CAT-1 extract. Absorbance at 280 nm (- - -), peroxidase activity (•-•-•), and catalase activity (—).

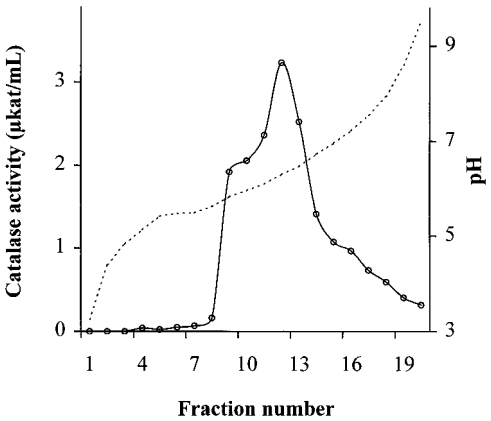

Figure 5. Isoelectrofocusing of C_{45d} extract: pH (- - -) and catalase activity (—○—).

on purified CAT-1 and CAT-2 extracts were unsuccessful due to a total loss of the CAT activity during the IEF experiment. Again, these results emphasize the poor stability of purified wheat germ CAT in low ionic strength solutions.

Determination of Molecular Weight. The molecular weight of each purified isoform of CAT from wheat germ has been determined by native-PAGE in gradient gels (10−15% in acrylamide). After revelation by a specific staining (Woodbury et al., 1971), the TSK extract, CAT-1, as well as CAT-2 exhibited only one active band with the same electrophoretic mobility as that of the bovine liver CAT present in the electrophoresis calibration kit of high molecular weight proteins. Therefore, a molecular weight of 240 kDa can be ascribed to the wheat germ catalases. CAT-1 and CAT-2 samples have also been revealed by protein staining in the same electrophoresis experiment. In addition to a protein band at 240 kDa, other bands were revealed, meaning that the CAT-1 and CAT-2 extracts are still contaminated by other proteins.

Effect of pH. The pH dependence of hydrogen peroxide dismutation catalyzed by the two isoforms, CAT-1 and CAT-2, is illustrated in Figure 6. Few differences were observed between these two catalases, and both exhibit an optimum pH close to pH 7. CAT-2 activity was almost not affected when the pH varied from 7 to 8 but rapidly decreased between pH 6.5 and 4.5. This latter phenomenon was more marked for the CAT-1 activity. At pH

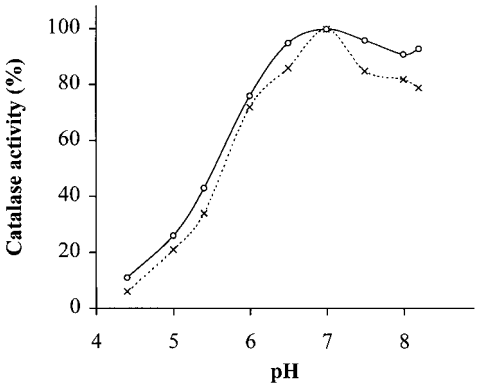

Figure 6. Effect of pH on the activity of CAT-1 (- -X- -) and CAT-2 (—○—).

Table 2. Effect of pH on the Kinetic Constants of the Wheat Germ Catalase Isoforms

		pH 5.6	pH 6.4	pH 7.0
CAT-1	K_m (M)	0.44	0.33	0.27
	V_m (μkat/mL)	0.48	1.18	1.39
	V_m/K_m (μkat/mL/M)	1.09	3.58	5.15
CAT-2	K_m (M)	0.30	0.43	0.45
	V_m (μkat/mL)	1.49	2.74	3.15
	V_m/K_m (μkat/mL/M)	4.97	6.37	7

4.5, the residual activities were less than 10 and 5% of the optimum activity for the CAT-2 and CAT-1 isoforms, respectively. Several authors have found similar results for plant catalases (Chan et al., 1978; Esaka and Asahi, 1982; Kieffer et al., 1982; Chandlee et al., 1983; Delcros et al., 1998). They indicated a pH optimum between 6 and 8 and a rapid decrease of the activity in the acidic part of the curve CAT activity vs pH.

The effect of pH was also studied on the kinetic constants of the two isoforms between pH 5.6 and 7. The values of K_m and V_m as well as those of V_m/K_m, which are related to the enzyme efficiency for low substrate concentrations, are given in Table 2. Both isoforms show a decrease of V_m when pH is decreased from 7 to 5.6, but the decrease is more marked for the CAT-1 isoform. Conversely, under the same conditions, a K_m decrease is observed for CAT-2 and the opposite, a K_m increase, for CAT-1. Thus, when the pH is decreased from 7 to 5.6, the decrease in efficiency (V_m/K_m) is less than 30% for the CAT-2 isoform, whereas it is close to 80% for the CAT-1 isoform. Therefore, in normal dough conditions where the pH is between 5.5 and 6.2 (Kieffer et al., 1981) and the hydrogen peroxide concentration is low (Liao et al., 1998), CAT-1 is probably much less efficient than CAT-2.

The stability of CAT-1 and CAT-2 at various pHs is shown in Figure 7. Incubation at 30 °C results in an inactivation when the pH is ≤6. The initial rate and the extent of inactivation at the end of the 3 h incubation are higher for CAT-1 than for CAT-2. Thus, at pH 5.4, 70% of the CAT-1 activity was lost in the first 5 min and the residual activity was less than 5% after 180 min of incubation, whereas for the CAT-2 activity, the initial (first 5 min of incubation) and final (180 min of incubation) losses were less than 30 and 60%, respectively. This inactivation is irreversible since dialysis against a buffer solution at pH 8 of the partially or totally inactivated fractions does not result in any recovery (neither total nor partial) of the initial activity. Similar results have been obtained for other plant catalases by Esaka and Asahi (1982) and Chan et al. (1978). The former authors showed that a 4 h incubation

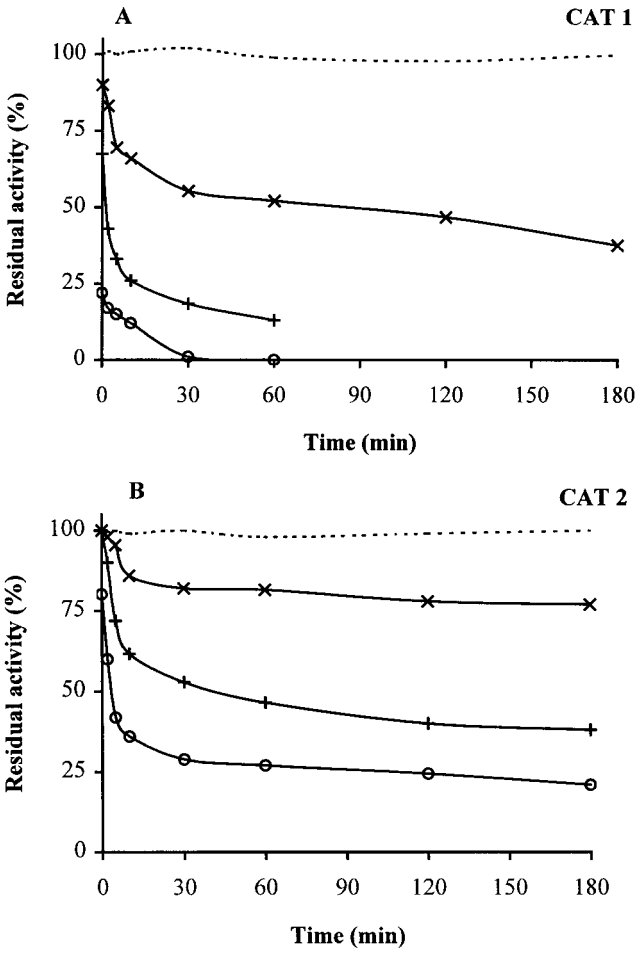

Figure 7. Stability of wheat germ catalase isoforms at various pHs. A: CAT-1 at pH 8 (- - -), pH 6 (—X—), pH 5.4 (—+—), and pH 5 (—○—) B: CAT-2 at pH 8 (- - -), pH 6 (—X—), pH 5.4 (—+—), and pH 5 (—○—).

at 4 °C and pH 5 of purified sweet potato root CAT resulted in a 80% loss of its activity. The latter authors showed that papaya CAT was irreversibly inactivated after 1 day of storage at 1 °C and at pH 5. They also indicated that the inactivation was total and irreversible after a 30 min exposure of the enzyme (whether purified or in puree) at pH 3.5 and 22 °C. According to Jones (1982), the inactivation is due to an irreversible dissociation of the prosthetic group from the apoprotein, and reconstitution of native, active CAT from free prosthetic group and apoprotein has never been achieved.

Our results concerning the wheat CAT sensitivity to acidic environment can be related to the findings of Delcros et al. (1998) and Rakotozafy et al. (1999). These authors found that, during dough mixing, the extractable CAT activity from dough decreased, and they showed that the CAT losses increased sharply when the dough was acidified either by adding acidic buffer solutions or glucose oxidase. According to theses authors, the latter addition resulted in the formation of δ-gluconolactone, which is slowly hydrolyzed in gluconic acid, acidifying the dough.

Inhibition Studies. 3-Amino-1,2,4 triazole (AT) has been known for a long time as a specific inhibitor of the CAT activity both in vivo and in vitro (Margoliash et al., 1960; Chang and Schroeder, 1972; Havir, 1992). It is assumed that this inhibitor binds irreversibly to the protein moiety of the enzyme (Margoliash et al., 1960; Chang and Schroeder, 1972). Several authors have

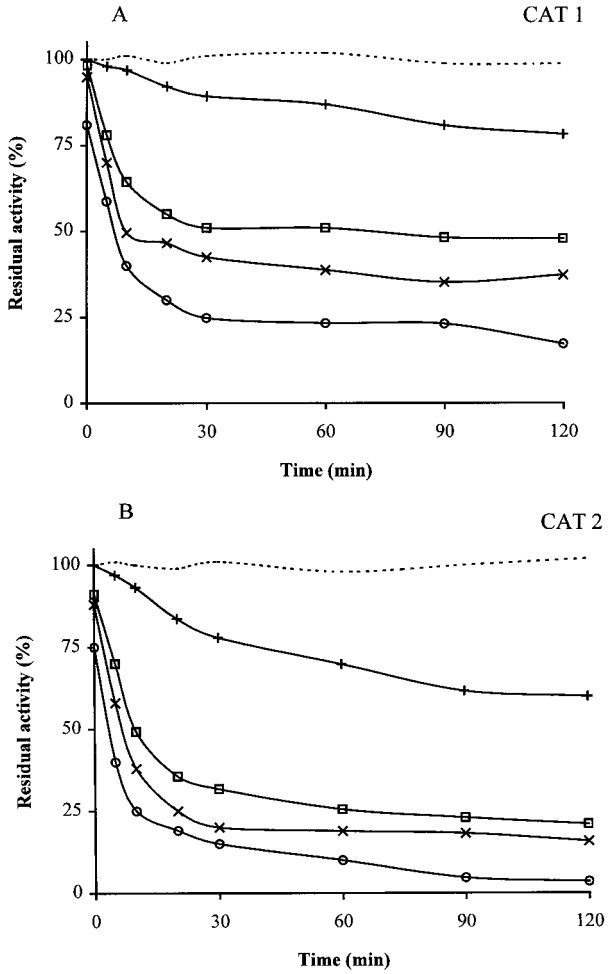

Figure 8. Stability of wheat germ catalase isoforms in the presence of 3-amino-1,2,4 triazole. A: CAT-1 with 0 mM (- - -), 0.15 mM (—+—), 7.5 mM (— — —), 15 mM (—X—), and 50 mM (—○—) of AT. B: CAT-2 with 0 mM (- - -), 0.15 mM (—+—), 7.5 mM (— — —), 15 mM (—X—), and 50 mM (—○—) of AT.

shown a specific and irreversible inhibition of catalases from various sources when the enzymes were incubated with the inhibitor for 1−2 h (Chandlee et al., 1983; Ferguson and Dunning, 1986; Havir, 1992). The time courses of inhibition of CAT-1 and CAT-2 by 0.15 to 50 mM AT are shown in Figure 8. At every AT concentration and for a similar incubation, the inhibition due to the incubation with AT was always less for CAT-1 than for CAT-2. Thus, after 2 h in the presence of 7.5 mM AT, the losses were close to 50 and 75% for the CAT-1 and CAT-2 activities, respectively. Similar results concerning the sensitivity to AT have been found for CAT isoforms isolated from maize (Chandlee et al., 1983) and from tobacco (Havir, 1992). According to Havir (1992), the differences in sensitivity to AT may be related to modifications in protein structure. We have verified the specificity of AT since a 2 h incubation of 50 mM AT with POD and LOX does not have any effect on their activity.

Guaiacol (2-methoxyphenol) is a synthetic substrate commonly used for POD assay. To study "in vitro" interactions between CAT and POD from wheat germ, the effect of this compound on CAT activity was tested. Experiments showed an increasing inhibition of the oxygen produced by CAT after addition of increasing amounts of guaiacol. The type of inhibition was deduced from Lineweaver−Burk double-reciprocal plots. A competitive inhibition pattern was found (Figure 9). The inhibition constants (K_i) were equal to 17 and 4 mM for CAT-1 and CAT-2, respectively, showing that CAT-2 was more sensitive to the inhibition by guaiacol than CAT-1. Guaiacol has no structural relation with hydrogen peroxide that could be raised to explain a competitive inhibition. Although we were unable to detect any change of coloration in the solution during the inhibition measurement, the inhibition could be related to the peroxidatic reactions catalyzed by catalases in the presence of hydrogen donors (Whitaker, 1985).

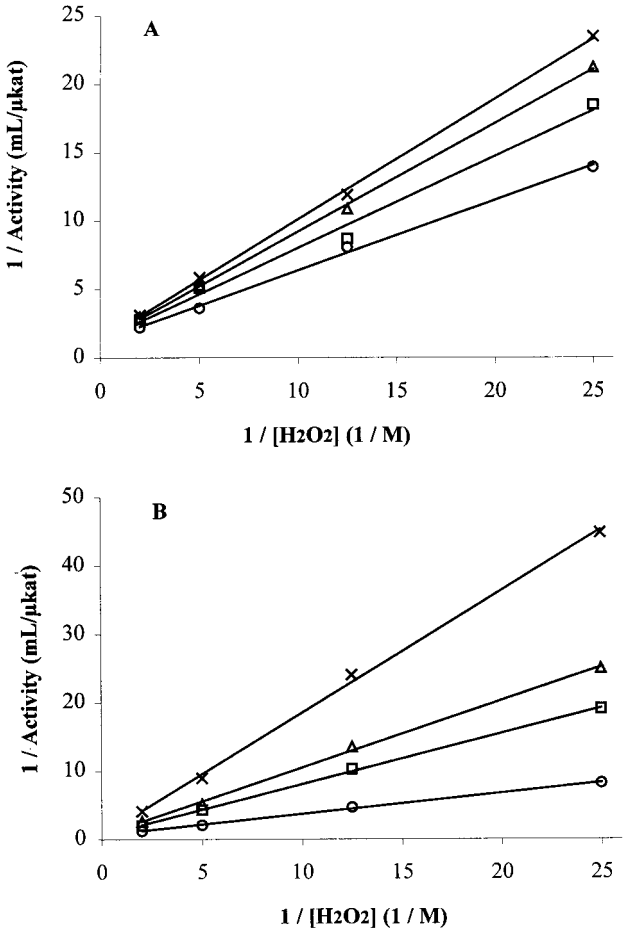

Figure 9. Inhibition of CAT 1 (A) and CAT 2 (B) by guaiacol.

CONCLUSION

The wheat germ is very rich in CAT activity. Two isoforms, CAT-1 and CAT-2, have been isolated from this source and can be used in further studies concerning the interactions among oxidoreducing enzymes from wheat and other sources during dough mixing. The low affinity of the CAT-1 isoform as well as its poor stability as soon as the pH is lower than 5.5 seems to indicate that its effect during dough mixing could be of less importance than that of the CAT-2 isoform.

ABBREVIATIONS USED

AS, ammonium sulfate; AT, 3-amino-1,2,4 triazole; C_{45}, precipitate of 45% saturation of AS; C_{45d}, dialyzed solution obtained from C_{45}; CAT, catalase; DEAE, diethylaminoethyl; IEF, isoelectric focusing; LOX, lipoxygenase; POD, peroxidase; S_{20}, supernatant of 20% saturation of AS; S_{45}, supernatant of 45% saturation of AS; TSK, Toyopearl-Butyl 650M (hydrophobic gel);

TSK_d, dialyzed fraction obtained from the TSK chromatography.

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