JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Characterization of a Peroxidase from Corn Steep Water

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Three cationic peroxidases have been detected in early, middle, and late corn steep water, with p*I* values of ∼8.9, ∼9.5, and >10.0. The major cationic corn steep water peroxidase (CSWP), with a p*I* >10, was purified 36400-fold with a 12% recovery from late steep water by a combination of acetone and ammonium sulfate precipitation and sequential chromatography on CM-cellulose, phenyl-Sepharose, and Sephadex G-75. The UV−vis spectrum of purified CSWP is typical of other plant class III peroxidases. The RZ (A_{403}/A_{280}) of CSWP was between 2.6 and 2.9. It is not glycosylated and exhibited an M_r of 30662 ± 7 by MALDI-TOF MS. The pH optimum of CSWP depends on the substrate, and it is active on 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), guaiacol, ferulic acid, *o*-dianisidine, *o*-phenylenediamine, and pyrogallol but is not active on either syringaldazine or ascorbate. At 75 °C and pH 4.5, the enzyme has half-lives of 22.7 min (0 mM Ca^{2+}) and 248 min (1 mM Ca^{2+}). The enzyme is stable at room temperature (22−25 °C), losing <3% of the activity at pH 4.5 and <10% at pH 6.2 over 400 h in the presence of 1 mM Ca^{2+}.

KEYWORDS: Cationic peroxidase; corn steep water; protein purification; characterization

INTRODUCTION

Peroxidases (EC 1.11.1.7) occur ubiquitously in nature (*1*, *2*). In the plant kingdom, they serve many purposes, including lignification, suberization and wound healing, protection against pathogen attack, and the scavenging of damaging hydrogen peroxide from the cell (*1*). The peroxidases are capable of oxidizing a broad variety of organic compounds including phenols, aromatic amines, indoles, and sulfonates using hydrogen peroxide as the oxidant (*3*−*5*). In the peroxidase reaction, hydrogen peroxide accepts two electrons during oxidation of the organic compound, the products being water and a free radical.

The peroxidases have become increasingly attractive ecologically benign catalysts capable of generating free radicals for the synthesis of a wide variety of polymers (*6*, *7*) (and references therein) for the stereospecific biotransformation of a variety of organic molecules (*3*−*5*), and in bioremediation (*8*−*12*). Many of these reactions are best run in organic solvents or in mixtures of aqueous and organic solvents. Among the many problems of using peroxidases in organic solvents is their limited solubility due to their proteinaceous nature. Moreover, because many of the peroxidases are glycosylated, solubility in organic solvents is further reduced by the hydrophilicity of the carbohydrate moieties.

Corn steeping is an integral part of the wet milling industry and serves to optimize recovery of starch from the corn kernel (*13*). Steeping involves the countercurrent movement of corn and steep water through a series of tanks, with the oldest corn meeting the freshest water and the freshest corn contacting the oldest water. The entire process, lasting between 30 and 50 h, is carried out at elevated temperature (48−52 °C) and low pH (3.7−4.3).

The composition of steep water is complex and contains substantial levels of lactic acid (produced by lactic acid bacteria), proteins and peptides (derived from both corn and bacteria) (*14*, *15*), carbohydrates (mostly derived from the corn) (*16*), and phytic acid and its hydrolysis products (*17*, *18*). Little is known about the enzymes present in corn steep water other than the presence of some glycohydrolases (α-amylase, *β*-galactosidase, *β*-hexosaminidase, and α-galactosidase) (*16*) and a peroxidase (personal communication, Professor C. D. Cox, Department of Microbiology, University of Iowa).

One of the objectives of our research is to add value to the byproducts and coproducts produced by agribusiness. Steep water, produced in vast amounts in the corn wet milling industry, represents a large, untapped resource for the identification and purification of valuable chemicals. In this paper, we describe the isolation, purification, and properties of a nonglycosylated cationic peroxidase from corn steep water (CSWP) that may be useful as an industrial biocatalyst.

MATERIALS AND METHODS

Samples of early, middle, and late steep water were obtained from a local industry and stored at 4 °C, at which temperature CSWP is unusually stable.

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10.1021/jf025883n CCC: $25.00
Published on Web 02/05/2003

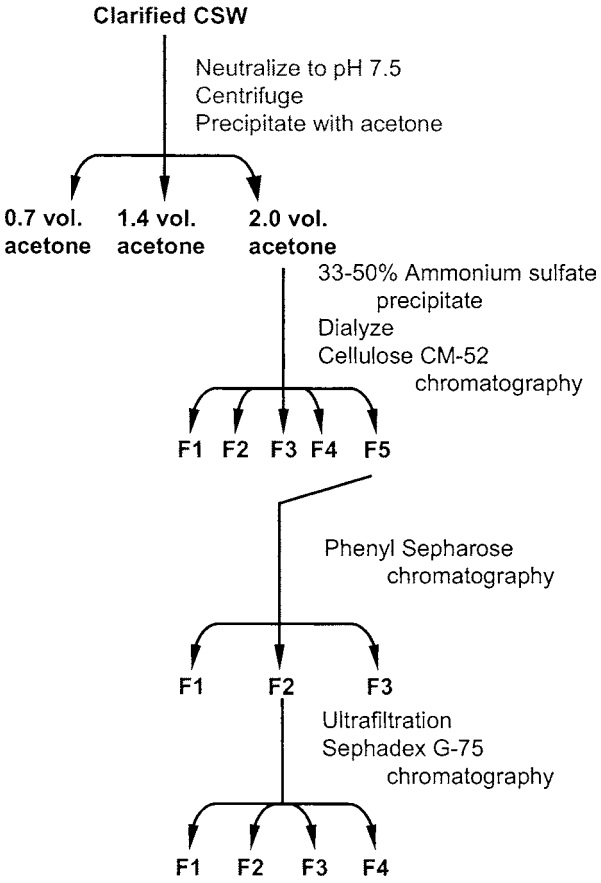

Figure 1. Scheme for the purification of CSWP from late steep water.

Clarification and Neutralization of Steep Water. The pH of steep water, clarified by centrifugation (30074*g*, 4 °C, 60 min), was adjusted to pH 7.5 with 50% (w/w) sodium hydroxide (Fisher Scientific, Fair Lawn, NJ). The precipitate was removed by centrifugation (30074*g*, 4 °C, 30 min) and discarded.

Purification of CSWP. The peroxidase was purified from late steep water as outlined in **Figure 1**. All precipitations with acetone and ammonium sulfate were performed at 4 °C, whereas the chromatography was carried out at room temperature (22−25 °C). All fractions from the various procedures were stored at 4 °C.

Cold acetone (700 mL/L neutralized steep) was added dropwise to give a flocculent white precipitate, which was allowed to settle for 30 min and discarded after removal by centrifugation (6174*g*, 4 °C, 10 min). Additional cold acetone (700 mL/L neutralized steep) was added, and a gummy precipitate, after settling for 30 min, was recovered by centrifugation as above. After removal of any residual acetone under a gentle stream of nitrogen, the precipitate was dissolved in a minimal volume of water and stored at 4 °C. Finally, cold acetone (600 mL/L neutralized steep) was added to the 1.4 (v/v) acetone supernatant, and a second gummy precipitate was recovered and treated as described above. Aqueous solutions of the 0.7−1.4 (v/v) and 1.4−2.0 (v/v) acetone fractions were clarified by centrifugation (30074*g*, 4 °C, 60 min).

Saturated (3.9 M) ammonium sulfate, pH 7, was added slowly to a stirred aqueous solution of the 1.4−2.0 (v/v) acetone fraction. The 33−50% saturated ammonium sulfate precipitate was recovered by centrifugation (30074*g*, 4 °C, 60 min), dissolved in a minimal volume of water, and dialyzed overnight against distilled water (2 × 4 L). The retentate, clarified by centrifugation, was passed over a CM-cellulose column (Whatman CM-52, 2.5 × 6.5 cm column equilibrated with 25 mM sodium phosphate buffer, pH 7.5). No pH adjustment of the retentate was necessary as the major peroxidase bound tightly to the column. Unbound and weakly bound proteins were washed from the column with equilibration buffer (~2 column volumes, 70 mL), and the column was eluted with a 400 mL linear NaCl gradient (0−250 mM) in equilibration buffer at a flow rate of 86 mL/h. Fractions (4 mL) were collected, and their A_{280} and A_{403} determined after appropriate dilution in water. Fractions were pooled according to their peroxidase activity, which was determined on 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide at pH 4.5 according to the standard protocol described below.

Sufficient saturated (3.9 M) ammonium sulfate, pH 7.0, was added to the major peroxidase peak to adjust the concentration to 0.975 M. After centrifugation, the sample was bound to a phenyl-Sepharose Fast Flow 6 column (Sigma, St. Louis, MO; 1.0 × 7.5 cm equilibrated with 0.975 M ammonium sulfate in 50 mM sodium phosphate buffer, pH 7.5). The column, after a washing with 50 mL of equilibration buffer, was developed with a 250 mL decreasing linear gradient from 0.975 to 0 M ammonium sulfate in 50 mM sodium phosphate buffer, pH 7.5, at a flow rate of 30.5 mL/h. Fractions (3.6 mL) were collected, and their A_{280}, A_{403}, and peroxidase activity determined as described above. Fractions with peroxidase activity were pooled according to their RZ values, which were concentrated to ~1 mL by ultrafiltration on an Amicon (Millipore Corp., Bedford, MA) YM10 Centriplus centrifugal filter.

The concentrated major peroxidase peak from the phenyl-Sepharose column was chromatographed on a Sephadex G-75 column (Sigma Sephadex G-75 superfine, 1.0 × 46 cm) equilibrated and eluted with water at a flow rate of 10.9 mL/h. Fractions (0.6 mL) were collected, and their A_{280}, A_{403}, and peroxidase activity determined as described above. Peroxidase-containing fractions, pooled according to their RZ values, were stored at 4 °C.

The purity of the final Sephadex G-75 preparation was determined by SDS-PAGE.

Gel Electrophoresis. SDS-PAGE was performed in 0.75 mm thick 12% (2.6% C) acrylamide gels in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA) as described by Laemmli (*19*) and stained for protein with Coomassie Blue R250 (0.1% Coomassie Blue R250 in 40% methanol/10% acetic acid for 2 h and destained until clear with 40% methanol/10% acetic acid). Native anionic PAGE was carried out using the same buffer system without SDS and 2-mercaptoethanol in the stacking, separating, and sample buffers. Gels were stained either for protein with Coomassie Brilliant Blue R250, as described, or for peroxidase activity with 3,3′-diaminobenzidine/urea hydrogen peroxide (Sigma Fast 3,3′-diaminobenzidine tablet sets) (Sigma).

Cationic gel electrophoresis was carried out in 0.75 mm thick 10% (2.6% C) acrylamide gels as described by Reisfeld et al. (*20*) (stacking gel, 0.0625 M potassium acetate, pH 6.8; separating gel, 0.375 M potassium acetate, pH 4.3; reservoir buffer, 0.35 M β-alanine−0.14 M acetate, pH 4.5), and the gels were stained either for protein or for peroxidase activity.

p*I* Determination. The p*I* of the CSWP was determined by PAGE in 0.75 mm thick 5% (2.6% C) acrylamide gels as described by Robertson et al. (*21*) with 25 mM sodium hydroxide and 20 mM acetic acid as the cathode and anode lock solutions, respectively. The pH gradient (useful range of 3.5−9.5) was formed by the incorporation of 2% Bio-Lyte 3/10 (Bio-Rad Laboratories) into the gel. Samples of purified soybean seed coat peroxidase isozyme c (SBPc, Bio-Research Products, Inc., North Liberty, IA), horseradish peroxidase isozyme c (HRPc type VI-A, Sigma), and horseradish peroxidase isozyme A2 (HRP5, Biozyme Laboratories International Ltd., San Diego CA) and a p*I* standard (IEF mix 3.6−9.3, Sigma) were run in parallel. After a brief wash in water, gels were stained for peroxidase activity. Ampholytes were removed from the gels by washing for 10 min in 10% (w/v) trichloroacetic acid and then overnight in 1% (w/v) trichloroacetic acid before staining with Coomassie Brilliant Blue R250.

The p*I* of the peroxidase was also determined on an agarose isoelectric focusing gel (pH 3−10, Whittaker Molecular Applications, Rockland, ME) and a polyacrylamide isoelectric focusing gel (Ampholine PAGplate pH 3.5−9.5, Pharmacia LKB Biotechnologies, Piscataway, NJ). Gels were run on an Isobox flatbed isoelectric focusing system (Hoefer Scientific Instruments, San Francisco, CA) at 5 °C and 900 V (15 W maximum) for 45 min with 1 M sodium hydroxide and 1 M phosphoric acid as the cathode and anode locks, respectively. After focusing, a vertical section of the gel containing the p*I* standards was excised, rinsed in water for 15 min, and stained for protein using the GelCode blue stain reagent (Pierce, Rockford, IL) as recommended

by the manufacturer. The remainder of the gel, after a 15 min rinse with water, was stained for peroxidase activity in a solution of 4.9 mM guaiacol−2.67 mM hydrogen peroxide in 0.2 M potassium phosphate buffer, pH 5.5. After the development of sufficient color, the reaction was stopped by soaking in water, and the gel was scanned.

Mass Spectrometry. The molecular weight of the purified CSWP was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Voyager DE STR BioSpectrometry workstation (Applied Biosystems, Foster City, CA). Samples (~1 pmol) in either α-cyano-4-hydroxycinnamic acid [a saturated solution in 50% acetonitrile−0.3% (v/v) trifluoroacetic acid] or sinapinic acid [10 mg/mL in 30% acetonitrile−0.1% (v/v) trifluoroacetic acid] were spotted on a gold plate using either the dried drop or the modified polycrystalline layer method as described previously (*22*). Instrument settings were as follows: accelerating voltage, 25 kV; grid voltage, 22.5 kV; guide wire, 12.5 V; extraction delay, 500 ns.

UV—Vis Spectra. Peroxidase samples were prepared in water to give an A_{403} of 0.53−0.55 and scanned between 240 and 700 nm on a UVICON 930 spectrophotometer (Kontron, Everet, MA) against a water blank.

Enzyme Assays, pH Optima, and Substrate Specificity. The standard peroxidase assay was performed at 25 °C with 1.67 mM ABTS and 1 mM hydrogen peroxide in 100 mM sodium acetate buffer, pH 4.5. The reaction mixture contained 980 μL of buffer plus ABTS (pre-equilibrated at 25 °C) and 1 or 10 μL of sample (up to 10 μg/mL peroxidase). The reaction was started with 10 μL of 100 mM hydrogen peroxide, and the change in absorbance at 414 nm was followed on a Gilford model 260 spectrophotometer and recorded on a PC fitted with a Keithley (Keithley Instruments, Inc., Cleveland, OH) MetraByte analogue input board, model ADC-16, and running the data acquisition program Gilford (kindly supplied by Dr. Frank Raushel, Department of Biochemistry, Texas A&M University, College Station, TX). The temperature was maintained at 25 °C with a Neslab Endocal model RTE9 refrigerated circulating water bath (Neslab Instruments, Inc., Portsmouth, NH).

The pH optimum for CSWP on a variety of reducing substrates was determined in McIlvaine buffers at pH 2.6−7.5 [prepared by mixing appropriate amounts of 0.05 M citric acid and 0.1 M sodium phosphate as described by Dawson et al. (*23*)]. All assays were run at 1 mM hydrogen peroxide and various reducing substrate concentrations as described under Results and Discussion. The following wavelengths and millimolar extinction coefficients were used in the assays: ABTS, 414 nm, $\epsilon = 36.0$ mM^{-1} cm^{-1} (*24*); guaiacol, 470 nm, $\epsilon = 26.6$ mM^{-1} cm^{-1} (*25*), $\epsilon = 5.2$ mM^{-1} cm^{-1} (*26*); *o*-phenylenediamine, 450 nm, $\epsilon = 11.1$ mM^{-1} cm^{-1} (*27*); ferulic acid, 318 nm, $\epsilon = 9.2$ mM^{-1} cm^{-1} (*28*); *o*-dianisidine, 460 nm, $\epsilon = 30.0$ mM^{-1} cm^{-1} (*29*); pyrogallol, 420 nm, $\epsilon = 2.6$ mM^{-1} cm^{-1} (*30*); syringaldazine, 525 nm, $\epsilon = 65.0$ (*31*); ascorbate, 290 nm, $\epsilon = 2.8$ (*32*). All assays (except with ferulic acid and ascorbate, which followed the decrease in reducing substrate concentration) recorded the increase in product concentration.

The reaction rate for each substrate at its pH optimum was converted to enzyme units (defined as the consumption/production of 1 μmol of substrate/product per minute at 25 °C and the pH of the assay) and used to evaluate the substrate specificity of the CSWP. Further details of each assay are presented below.

Stability of CSWP. The effect of pH and Ca^{2+} on the heat stability of CSWP was determined by incubating the enzyme (10 μg/mL, 50 μL) in prelubricated 650 μL microfuge tubes (Costar, Corning, Inc., Corning, NY) for 1 min and for 60 min at 75 °C in 25 mM sodium acetate buffer, pH 4.5, or in 25 mM Bis-Tris buffer, pH 6.2, with and without 1 mM Ca^{2+}. Residual peroxidase activity in the samples was assayed on ABTS and hydrogen peroxide (standard assay) after allowing refolding to proceed for either 30 min or 20 h at 4 °C. The initial 1 min of heating gave rise to more reproducible data, probably due to compensation for factors such as adsorption of enzyme to the plastic surfaces of the microfuge tube. The loss of activity due to heat denaturation in 1 min of heating was calculated to be <5% in the absence of Ca^{2+} and <0.5% in the presence of 1 mM Ca^{2+}.

The heat stability of CSWP at 75 °C was determined in 25 mM sodium acetate buffer, pH 4.5, with and without 1 mM Ca^{2+}. Microfuge tubes with CSWP (10 μg/mL, 50 μL) were set up as above and incubated for various periods of time at 75 °C in a water bath. Periodically, tubes were removed and assayed for peroxidase activity on ABTS and hydrogen peroxide (standard assay) after incubation at 4 °C for 30 min and for 15 h.

Curves were analyzed using the equation for single-exponential decay by plotting the natural logarithm of remaining peroxidase activity [$\ln(A_t/A_0)$, where A_t = activity remaining at time = t and A_0 = activity at time = 0] against time and determining the slope by linear regression in an Excel spreadsheet (Microsoft, Redmond, WA). The half-life, $t_{1/2}$, is given by the expression, ln 2/slope, or 0.693/slope with units of min. The data were also fitted to a single-exponential decay equation by nonlinear least-squares regression using Enzfitter (BioSoft, Ferguson, MO). The results from both fits agreed well with each other.

Stability at room temperature was determined by incubating CSWP (10 μg/mL, 250 μL) in either 25 mM sodium phosphate buffer, pH 4.5, or 25 mM Bis-Tris buffer, pH 6.2 (each supplemented with 0 or 1 mM Ca^{2+}), at room temperature (22−24 °C) for 400 h on the bench. Toluene (5 μL) was added to each tube to control bacterial growth. Samples were removed periodically and assayed for residual peroxidase activity on ABTS and hydrogen peroxide.

Amino Acid Composition. CSWP (20 μg) was hydrolyzed with 6 N hydrochloric acid−1% phenol (100 μL) at 110 °C for 24 h under an argon atmosphere with norleucine as an internal standard. After removal of the hydrochloric acid on a SpeedVac (Thermo Savant, Holbrook, NY), the sample was dissolved in sample buffer and analyzed on a Beckman (Beckman Coulter, Inc., Fullerton, CA) model 6300 amino acid analyzer with a standard three sodium citrate buffer/three temperature protocol and using System Gold to collect and analyze the data. An amino acid standard (Beckman-Coulter 7300/6300 amino acid standard), hydrolyzed in parallel under exactly the same conditions, was analyzed with the unknown, allowing some compensation for loss of the amino acids, particularly serine and threonine [estimated losses are about 5 and 10%, respectively (*33*)]. These manipulations do not correct for any underestimation of leucine, isoleucine, and valine.

Methionine and cysteine were analyzed and quantified as methionine sulfone and cysteic acid, respectively, after overnight oxidation with performic acid (*34*). An amino acid standard, treated identically in parallel, was used to calibrate the amino acid analyzer.

Protein Assay. Protein was assayed using the Micro BCA protein assay kit (Pierce, Rockford, IL) or by the Bradford method (*35*) using the kit supplied by Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin was used as a standard.

Carbohydrate Analysis. Carbohydrate was determined in CSWP by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a DX 500 chromatography system (Dionex Corp., Sunnyvale, CA) as described by Hardy and Townsend (*36*). Briefly, CSWP (10 μg), SBPc (10 μg), and a water blank were hydrolyzed with 2 M trifluoroacetic acid at 121 °C for 2 h in 2 mL screw-cap glass vials sealed with a Teflon-faced septum. After hydrolysis, the vials were cooled and centrifuged briefly to collect any condensate in the bottom of the vial; the trifluoroacetic acid was evaporated under a gentle stream of nitrogen at 40 °C. Complete removal of the trifluoroacetic acid was ensured by repeated evaporation from 2-propanol (2 × 100 μL) and from water (3 × 100 μL). The samples, dissolved in 500 μL of water, were filtered through a 0.45 μm Millex filter and analyzed on a CarboPac PA1 column eluted isocratically with 16 mM sodium hydroxide. A standard containing fucose, glucosamine, galactose, glucose, and mannose was analyzed before and after the glycoprotein samples.

RESULTS AND DISCUSSION

Purification of CSWP. Steep water is produced in enormous quantities during the steeping of corn and is the potential source of many value-added products such as enzymes, which can be used to offset the cost of processing the steep into other, high-volume, lower value products such as fermentation media and animal feeds.

Peroxidase activity was detected in samples drawn from the early, middle, and late steeps and was highest in the late steep,

with ~1 unit/mL being detected when assayed at pH 4.5 on ABTS and hydrogen peroxide. The activity was considerably lower in the middle and early steeps, being about 60 and 30%, respectively, of that in the late steep. The late steep was chosen for the purification of a cationic peroxidase, not only because it contained the highest peroxidase activity of the steeps assayed but also because of its contact with the freshest corn. The latter is an important consideration because the corn is the probable source of the peroxidase.

CSWP was purified from the late steep by a combination of acetone and ammonium sulfate precipitations and column chromatography on CM-52 cellulose cation-exchange resin, phenyl-Sepharose, and Sephadex G-75 (**Figures 1** and **2**; **Table 1**).

The initial adjustment of the pH of the steep water to pH 7.5 gave rise to a heavy precipitate (~92 g L^{-1}) with little loss of protein (<10%) and minimal loss of peroxidase activity (**Table 1**).

Capture of the peroxidase by ion exchange resins requires the removal of a large part of the salts and lactic acid, a task conveniently accomplished by ultrafiltration. However, attempts to reduce the volume of the neutralized steep by ultrafiltration at this stage were unsuccessful due to rapid fouling of the filter. An alternative process, namely, dialysis of the neutralized steep water and subsequent capture by ion exchange, was tedious and time-consuming due to the volumes involved. Because preliminary experiments showed that excellent recoveries with reasonable purification and a large reduction in volume could be obtained through acetone precipitation, this approach was used in the present study.

Acetone precipitation and collection of the 1.4−2.0 (v/v) precipitate resulted in large reductions in the volume (from ~4000 to 290 mL) and protein content (from 138.9 to 20.3 g), with a purification and recovery of peroxidase activity of 4.8-fold and 60.0%, respectively (**Table 1**). The overall recovery of peroxidase activity in the 0.7−1.4 and 1.4−2.0 (v/v) acetone precipitates was 95.2%.

The 33−50% saturated ammonium sulfate precipitate obtained from the 1.4−2.0 (v/v) acetone fraction, after dialysis, resulted in a substantial increase in purity of the peroxidase (74.1-fold) (**Table 1**). Less than 2% of the peroxidase activity remained in the 0−33 and 50−60% saturated ammonium sulfate fractions.

Four peaks of peroxidase activity were observed after chromatography of the dialyzed 33−50% saturated ammonium sulfate fraction on CM-52 cellulose (**Figure 2A**) and were pooled into five fractions (CM-52/F1−F5). A minor part of the peroxidase activity (CM-52/F1), representing ~2% of the total activity, did not bind to the column and eluted as a broad peak in the unbound volume. About 1.2% of the total peroxidase activity bound weakly to the column and eluted as a sharp peak (CM-52/F2) in the starting buffer. Two peaks of peroxidase activity eluted close to 50 mM NaCl and were fractionated such that CM-52/F3 contained the leading edge and apex of the minor peak, CM-52/F4 the trailing edge of the minor peak and the leading edge of the major peak, and CM-52/F5 the bulk of the major peak (**Figure 2A**). These fractions represented 13.0, 10.6, and 68.7%, respectively, of the total peroxidase activity loaded onto the column. Purification of CM52/F3, CM52/F4, and CM52/F5 at this stage was 376-, 557-, and 4300-fold, respectively. Total recovery of peroxidase activity from the column was excellent, with total activity across all pooled fractions summing to >100% of that applied to the column.

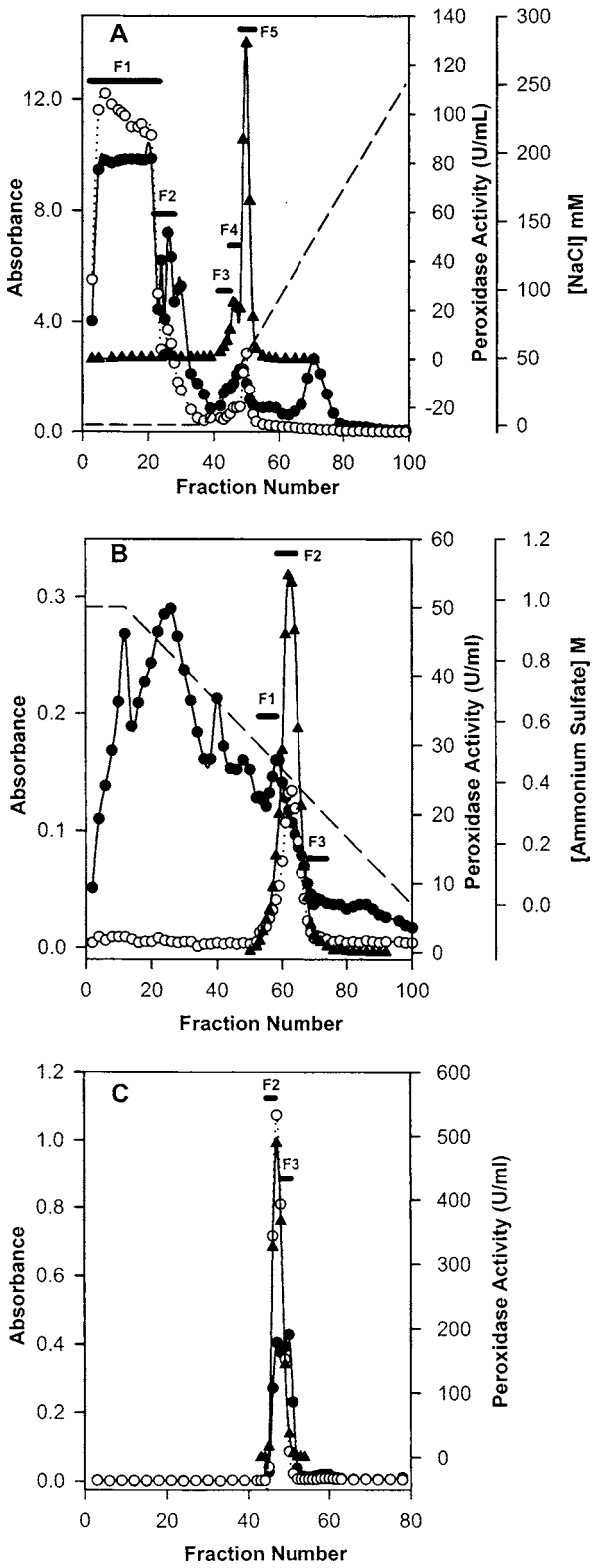

Figure 2. Column chromatography of CSWP: (A) CM-52 cellulose; (B) phenyl-Sepharose 6 Fast Flow; (C) Sephadex G-75. Symbols are consistent throughout the panels: (●) A_{280}; (○) A_{403}; (▲) peroxidase activity (units/mL); (– –) [NaCl] (mM) (A) and [$(NH_4)_2SO_4$] (M) (B). Details of the chromatography are presented in the text. Some of the axes have been offset to improve legibility. Bars indicate the pooled fractions.

Chromatography of CM-52/F5 on phenyl-Sepharose gave rise to a broad peak of peroxidase activity (**Figure 2B**), which was split into three pools on the basis of the RZ values of the fractions. Moving across the peak, PS/F1, PS/F2, and PS/F3

Table 1. Purification and Recovery of CSWP from Late Corn Steep Water

sample	volume (mL)	protein (mg/mL)	peroxidase (units/mL)	specific activity (units/mg)	purification (cumulative yield %)
LSW before neutralization	3800	39.45	1.01	0.03	1.0 (100.0)
LSW after neutralization	3800	36.56	0.99	0.03	1.1 (98.0)
1.4–2.0 volume acetone fraction	290	70.13	7.94	0.12	4.8 (60.0)
33–50% ammonium sulfate precipitate after dialysis	111	10.70	20.29	1.90	74.1 (58.7)
CM-52/F5	24.20	0.65	71.90	110.28	4307.0 (45.3)
phenyl-Sepharose F2	21.60	2.91	44.30	314.73	12292.2 (24.9)
G-75/F1	0.57	–[a]	16.19	–	– (2.4)
G-75/F2	1.16	0.44	413.74	931.85	36394.6 (12.5)
G-75/F3	1.19	0.45	258.79	575.09	22460.9 (8.2)

[a] Protein <10 μg/mL.

contained fractions with RZ ranging from 0.05 to 0.5, from 0.8 to 1.2, and from 0.2 to 0.6, respectively. Overall recovery from this step was 84.1%, with PS/F2 representing 57.0% of the activity loaded on the column. It is important to keep the concentration of ammonium sulfate below that (<33% saturation) used above for precipitation of the peroxidase from the acetone fractions. Improved recovery of the cationic peroxidase can be obtained by pooling PS/F1 and PS/F3 for rechromatography on the phenyl-Sepharose column. The three fractions from the phenyl-Sepharose column were concentrated on an Amicon Centriplus YM-10 ultrafilter (10 kDa MWCO).

A single, sharp peak of peroxidase activity overlapping two incompletely resolved A_{280} peaks was observed after chromatography of PS/F2 on Sephadex G-75 (**Figure 2C**). RZ values were used to combine tubes containing peroxidase activity into four fractions, G-75/F1−F4. The major fraction, G-75/F2, was purified ~36400-fold with an overall yield of ~12% of the activity in the starting material (**Table 1**).

Chromatography on phenyl-Sepharose was important despite the lower recovery of pure CSWP due to the conservative pooling of fractions, because bypassing this step. that is, chromatography of the CM-52/F5 fraction directly on Sephadex G-75, gave a final RZ value of ~1.0, which is significantly lower than that obtained for pure CSWP (RZ = 2.6−2.9).

The low recovery of CSWP is not due to inactivation or irreversible binding of the peroxidase to the different chromatography media. About 480 units (400 μg) of purified CSWP was obtained from 3838 units (3800 mL of late steep water). The peroxidase activities of all the fractions recovered and not purified further sum to 2860 units, giving an overall recovery of peroxidase activity (inclusive of pure CSWP) of 84.4%. In retrospect, recovery of pure CSWP can be greatly improved by modifying the precipitation and chromatographic steps and by less conservative pooling of fractions, For example, broader acetone cuts can be used as well as binding the enzyme to CM-cellulose at higher pH. The modification of gradients in the ion exchange and phenyl-Sepharose chromatographies will also improve recoveries of CSWP. The purity of the CSWP required will depend on the enzyme application, purer enzyme being necessary for diagnostic and analytical use and lower purity for other uses such as in bioremediation. Thus, impure fractions from different parts of the purification protocol reported here may suffice for many applications of CSWP.

These data allow the estimation that CSWP is present in the late steep at a concentration of ~0.7 mg/L with other peroxidases contributing another 0.2 mg/L (calculations based on the recovery of CSWP, which represents ~80% of the total peroxidase activity).

CSWP from two independently collected batches of late steep water was purified by this procedure with similar results, with the RZ value of the first preparation being 2.9 and that for the second being 2.6. SDS-PAGE analysis of the purified CSWP revealed the presence of a single major band migrating with an M_r of ~33000 (not shown).

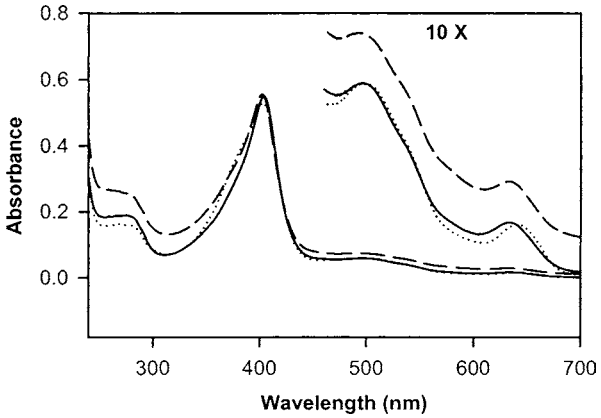

Figure 3. UV–vis scan of CSWP (—), SBPc (– –), and HRPc (•••). Peroxidases were dissolved in water to the same A_{403} and scanned between 240 and 700 nm. The absorbance scale is expanded 10-fold between 460 and 700 nm (inset).

All characterization of CSWP reported here was carried out on G-75/F2 from the first enzyme purification.

UV—Vis Spectra. The UV−vis spectrum of CSWP in water is similar to those of soybean seed coat peroxidase (SBPc) and horseradish peroxidase isozyme c (HRPc) recorded under the same conditions. A Soret band is observed at 403.5 nm, and α- and β-bands are seen at 496 and 633 nm, respectively. These values are similar to those observed for SBPc (402, 490.5, and 633 nm, respectively) and HRPc (402.5, 498, and 641 nm, respectively) (**Figure 3**) and to those of other class III peroxidases reported in the literature such as an anionic tobacco peroxidase (*29*), barley grain peroxidase, BP 1 (*37*), and royal palm tree peroxidase (*26*).

The millimolar extinction coefficient at 403 nm was determined to be 115.8 mM^{-1} cm^{-1} (protein quantitation by amino acid analysis, see below), assuming one heme group per enzyme molecule. This value compares well with the millimolar extinction coefficients reported for the basic peroxidases from horseradish (*38*), which range from 111.3 to 115.9 mM^{-1} cm^{-1}, for the basic peroxidase from tea of 115 mM^{-1} cm^{-1} (*39*), and for the secreted anionic and cationic peroxidases from peanut cell culture of 112 mM^{-1} cm^{-1} (*40*), but differ for those from the neutral and acidic peroxidases from horseradish, which range from 97 to 104 mM^{-1} cm^{-1} (*38*, *41*).

Amino Acid Composition and Carbohydrate Analysis. The amino acid composition of CSWP is within the range of the cationic peroxidases from horseradish [E3−E6 (*38*)], turnip P7

Table 2. Amino Acid Composition of CSWP and Its Comparison to the Cationic Peroxidase Isozymes from Horseradish (E3–E6) and Turnip Peroxidase Isozyme P7 and the Neutral Horseradish Peroxidase Isozyme c

	mol %	mol/mol of protein						
residue	CSWP	CSWP	HRP E3	HRP E4	HRP E5	HRP E6	HRPc	turnip P7
Asx	10.73	30.1	38.0	37.5	44.4	41.5	48	39
Thr	7.23	20.3	23.2	23.9	23.6	27.9	25	16
Ser	9.77	27.3	22.8	22.8	25.4	29.2	25	42
Glx	6.83	19.1	20.9	20.9	16.2	16.9	20	14
Pro	4.63	13.0	14.8	14.4	19.2	11.4	17	11
Gly	10.14	28.4	30.6	30.4	20.4	23.0	17	24
Ala	10.86	30.4	23.0	22.7	25.6	32.1	23	32
Cys	2.45	6.9	8.1	7.9	7.8	8.1	8	8
Val	5.63	15.8	27.1	28.2	20.0	19.0	17	19
Met	1.78	5.0	4.1	3.8	3.7	4.7	4	6
Ile	3.88	10.9	15.4	16.0	14.2	15.7	13	15
Leu	9.76	27.3	24.4	24.3	34.0	21.5	35	21
Tyr	1.73	4.8	2.1	2.1	5.3	4.1	5	4
Phe	4.72	13.2	12.9	13.2	20.9	14.3	20	14
His	1.00	2.8	4.1	4.0	3.1	3.0	3	3
Lys	1.06	3.0	5.6	5.2	7.3	7.2	6	10
Arg	7.78	21.8	24.0	24.5	25.7	22.7	21	17

(*42*, *43*), and HRPc (*44*) except for lower levels of aspartic acid/asparagine and lysine (**Table 2**). The ratio of acidic amino acid residues and their amides (Asx + Glx) to the basic amino acid residues (Arg + Lys) of CSWP (based on the mole percent values) is 1.98 and is similar to those of the cationic horseradish peroxidases E3, E4, E5, and E6 (1.99, 1.97, 1.83, and 1.95, respectively) (*38*) and turnip TP7 cationic peroxidase (1.96) (*42*, *43*). This ratio is considerably lower than those calculated for the anionic peroxidases from soybean seed coat, SBPc (4.00) (*45*), horseradish A2, *Arabidopsis* ATP-A2 (4.60 and 4.40, respectively) (*46*), and sycamore maple (*Acer pseudoplatanus*) (4.04) (*47*). Clearly, this ratio is significant in determining the p*I* of the enzyme, although, ultimately, the extent of amidation of the Asp and Glu residues must also be critical.

The presence of at least seven cysteine residues in CSWP clearly differentiates this peroxidase from the class I peroxidases (bacterial and ascorbate peroxidases), in which this amino acid is present at much lower levels. No cysteic acid or methionine sulfone was present in the acid hydrolyzate of the CSWP sample before performic oxidation, indicating that no oxidation of these amino acids occurred in the late steep.

Unlike most class III plant peroxidases, CSWP is not glycosylated as no traces of fucose, glucosamine, or mannose were found after hydrolysis of CSWP with 2 M trifluoroacetic acid and HPAEC-PAD analysis. These sugars were all detected in the hydrolyzed SBPc sample that was used as a positive control. Another rare example of a nonglycosylated class III peroxidase is the barley seed variant, BP 1b, which comprises ~33% of the total BP 1 pool (*48*).

Molecular Weight and p*I*. The M_r of CSWP was found to be ~33000 by SDS-PAGE (data not shown) and 30662 ± 7 [mean of six determinations ± SE (mean)] by MALDI-TOF MS (**Figure 4**, top). Strong signals were observed in the MALDI spectrum for both the singly charged species ($[M + H]^+$) and for the doubly charged species ($[M + 2H]^{2+}$). Also present in the spectrum are minor signals, representing <2% of the total signal, from contaminating proteins at *m*/*z* 26896 and 22038. Both contaminants, together with CSWP, are major species in G-75/F3 (**Figure 4**, bottom).

The relatively low M_r of CSWP (30662) reflects the absence of glycosylation, but partial degradation of the peroxidase in the complex environment of the steep cannot be excluded.

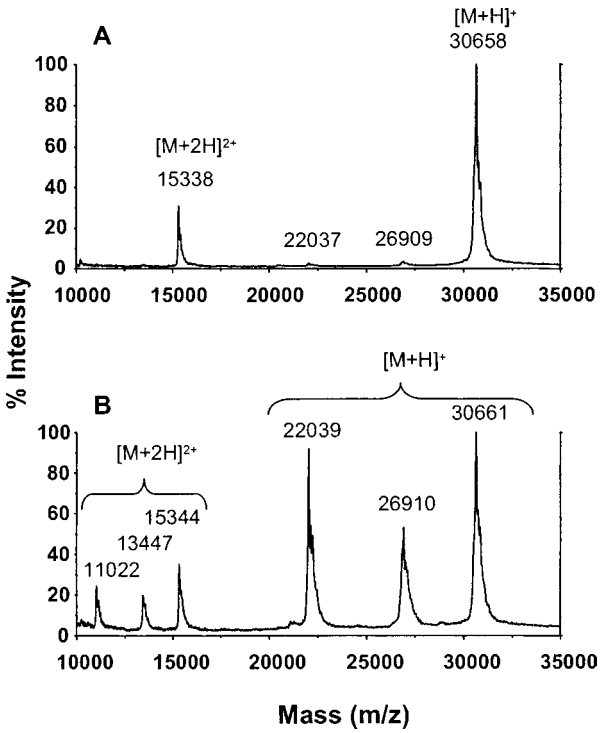

Figure 4. MALDI-TOF MS analysis of CSWP fraction from Sephadex G-75 fractions F2 (A) and F3 (B). Samples in sinapinic acid were deposited as dried drops, allowed to air-dry, and analyzed with the following instrument settings: acceleration, 25 kV; grid voltage, 22.5 kV; guide wire, 12.5 V; extraction delay, 500 ns.

However, this is unlikely to be a major factor because the high protein content of the steep, simply by competition for any proteases, would exert a protective effect.

Isoelectric focusing according to the method of Robertson (*21*) was unsuccessful, and CSWP was not observed when the sample was applied to either the cationic or anionic sides of the gel (data not shown). This suggested that CSWP did not enter the gel when loaded on the cationic side and ran off the gel when loaded on the anionic side. This was confirmed when isoelectric focusing was performed on polyacrylamide and agarose gels on a Hoefer Isobox horizontal electrophoresis unit, where CSWP migrated right up against the wick soaked in the cationic solution (1 M NaOH). It therefore appears that the p*I* of CSWP is >10 and therefore too high to be determined by commercially available ampholytes. It joins a list of other cationic peroxidases with high p*I* values such as the basic horseradish peroxidase enzymes E3−E6, p*I* >12 (*38*), and turnip isozyme P7, with a p*I* of 11.6 (*42*).

Two additional minor peroxidases were observed in the 50% saturated ammonium sulfate fraction, one migrating with a p*I* a little ahead of that of HRPc (p*I* ~8.8) and one migrating with a p*I* >9.5 (data not shown).

pH Optima and Substrate Specificity. The pH optimum of CSWP is substrate-dependent (**Table 3**). On ABTS, there is a sharp pH optimum (pH 3.4), with ~20% of the activity remaining at pH 4.5 (the standard pH of the assay on ABTS used in this study). A broader, acidic pH optimum is found for the activity of CSWP on ferulic acid (3.6−4.6). The low pH optima of CSWP on these two substrates are similar to that found for the barley seed peroxidase, BP 1 (*37*, *49*), and for a peroxidase from *Vaccinium myrtillus* (bilberry) (*50*).

CSWP has a pH optimum of 4.6 on guaiacol but is atypical in that the activity is within 90% of the maximum over a broad pH range (3.7−5.2). The high activity of CSWP on guaiacol at

Table 3. pH Optima and Activity of CSWP on Different Substrates (Assay Conditions and References Are Presented in the Text)

substrate	wavelength (nm)	substrate concentration (mM)	millimolar extinction coefficient (mM^{-1} cm^{-1})	pH optimum	specific activity (units/mg of CSWP)
ABTS	414	1.67	36.0	3.4	4615.0
guaiacol	470	5.83	26.6	3.7–5.2	734.7
			5.2		3758.3
o-phenylenediamine	450	0.25	11.1	5.2	340.2
ferulic acid	318	0.098	9.2	3.6–4.6	3774.0
o-dianisidine	460	0.51	30.0	5.2	2983.8
pyrogallol	420	50.0	2.6	5.6–6.8	456.6
syringaldazine	525	0.25	65	no activity	no activity
ascorbate	290	0.40	2.8	no activity	no activity

pH <4 is unusual and differs from other peroxidases, including those from royal palm (*26*), *V. myrtillus* (bilberry) (*50*), and the anionic, neutral, and cationic peroxidases from turnip (*51*, *52*), for which the optima are closer to pH 5 and the activity decreases rapidly below pH 4.5.

The pH optima determined on *o*-phenylenediamine, *o*-dianisidine, and pyrogallol are in the range of 5.2−6.8 and are more typical of other class III peroxidases reported in the literature.

It was difficult to determine the activity of CSWP on pyrogallol, with high pyrogallol concentrations being necessary. At pH >7.2, autoxidation of pyrogallol proceeded rapidly in the absence of enzyme and hydrogen peroxide. Moreover, at pH >6, CSWP, in the absence of hydrogen peroxide, increased the oxidation rate of pyrogallol. Thus, the determination of peroxidase activity required correction for the activity of CSWP on pyrogallol in the absence of hydrogen peroxide. It was also found that the assay was best started by the addition of enzyme to the cuvette rather than by the addition of hydrogen peroxide.

CSWP is completely inactive on syringaldazine [a lignin analogue frequently used to determine if a peroxidase is involved in lignin and secondary cell wall biosynthesis (*53*−*55*)] and on ascorbate.

The specific activity of CSWP on ABTS, ferulic acid, *o*-dianisidine, guaiacol, pyrogallol, and *o*-phenylenediamine decreases in this order (**Table 3**), which is, however, uncertain and depends on the millimolar extinction coefficient used for guaiacol oxidation. Using the value (5.2 mM^{-1} cm^{-1}) reported for guaiacol oxidation by Sakharov (*26*), a specific activity of 3758.3 units/mg of CSWP can be calculated, and the order now becomes ABTS > guaiacol > ferulic acid > *o*-dianisidine > pyrogallol > *o*-phenylenediamine. A value of 8.64 mM^{-1} cm^{-1} for guaiacol oxidation has been determined by Dr. Bryce Cunningham (Bio-Research Products, North Liberty, IA) using hydrogen peroxide accurately standardized with ceric sulfate (personal communication), a value closer to that reported by Sakharov et al. (*26*) than that reported by Chance (*25*). More recently, Doerge et al. (*56*) have shown that a dimer, 3,3′-dimethoxy-4,4′-biphenoquinone (λ_{max} 412 and 470 nm), together with minor amounts of 3,3′-dimethoxy-4,4′-dihydroxybiphenyl (its reduced precursor with no visible absorbance), rather than tetraguaiacol, are the initial products of guaiacol oxidation by lactoperoxidase. The major product is not completely stable, and the A_{470} starts to decrease after ∼30 s, declining ∼10% in 180 s. This instability is not too problematical, provided a time limit for the assay is established, because enzyme assays are set up to measure initial rates of reaction. It is clear that a more representative value for the molar extinction coefficient of the guaiacol oxidation product(s) would be useful and worth determining.

CSWP, in common with other peroxidases containing iron-protoporphyrin IX (*39*, *52*, *57*), is inhibited by sodium cyanide and by sodium azide with the inhibition by cyanide being more severe (100% at 10 μM) than with azide (30 and 15% at 10 and 1 μM, respectively).

Stability of CSWP. CSWP is stable at room temperature (22−25 °C) at pH 4.5 and 6.2 with ∼7% of the activity lost over 400 h at pH 4.5 and ∼15% at pH 6.2. In the presence of 1 mM Ca^{2+}, the stability is increased, with <3% loss of activity at pH 4.5 and <10% at pH 6.5.

The protection of CSWP by 1 mM Ca^{2+} is more dramatic at elevated temperatures, and nearly 80% of the peroxidase activity is recovered at both pH 4.5 and 6.2 after 60 min at 75 °C and 30 min on ice, with little increase in peroxidase activity after continued incubation for 20 h at 4 °C. In contrast, the recovery of peroxidase activity in the absence of Ca^{2+} is 14.4% at pH 4.5 and 31.1% at pH 6.2 after 60 min at 75 °C and 30 min at 4 °C, which, after 20 h at 4 °C, increased to 36.1% (a 2.5-fold increase) at pH 4.5 and to 42.2% (a 1.4-fold increase) at pH 6.2.

Thus, the final recovery of peroxidase activity depends on the rate of refolding of the denatured enzyme, which is influenced by the presence of Ca^{2+} and by pH. In the absence of Ca^{2+}, refolding of the peroxidase is slower at pH 4.5 than at pH 6.2, requiring 20 h for the activity at pH 4.5 to recover to levels similar to those observed after 30 min at pH 6.2. The presence of Ca^{2+} essentially abolishes this pH effect, and similar recoveries of activity are observed at both pH 4.5 and 6.2 after 30 min of refolding.

The protective effect of Ca^{2+} at pH 4.5 is also observed on the half-lives of CSWP (**Figure 5**) incubated in the presence or absence of 1 mM Ca^{2+}. After 30 min of refolding, $t_{1/2}$ is found to be 22.7 min in the absence of Ca^{2+} and 248 min in the presence of 1 mM Ca^{2+}. After 15 h at 4 °C, the $t_{1/2}$ had, in the absence of Ca^{2+}, increased 1.6-fold to 35.2 min with no concomitant increase (but rather a decrease from 248 to 216 min) in the $t_{1/2}$ in the presence of 1 mM Ca^{2+} (**Figure 5**). These results confirm those reported above for the single time point experiment.

The active site of plant peroxidases has a heme group that can be removed under acidic conditions (*58*), leading to complete inactivation of the enzyme. Reincorporation of the heme group requires an increase in pH and in heme concentration, the presence of a protein denaturant, such as urea, and the correct oxidation/reduction potential, often provided by a mixture of reduced and oxidized glutathione [see Smith et al. (*59*) for an example of the conditions necessary for peroxidase refolding]. Thus, specific conditions are necessary for the efficient refolding and heme incorporation by peroxidases that are not met in the experiments reported here. Plant and fungal peroxidases (lignin peroxidases) normally bind two calcium ions, one at the proximal end and one at the distal end of the enzyme (*2*, *3*, *60*). A major role for these bound calcium ions appears to be the maintenance of the protein structure around the heme

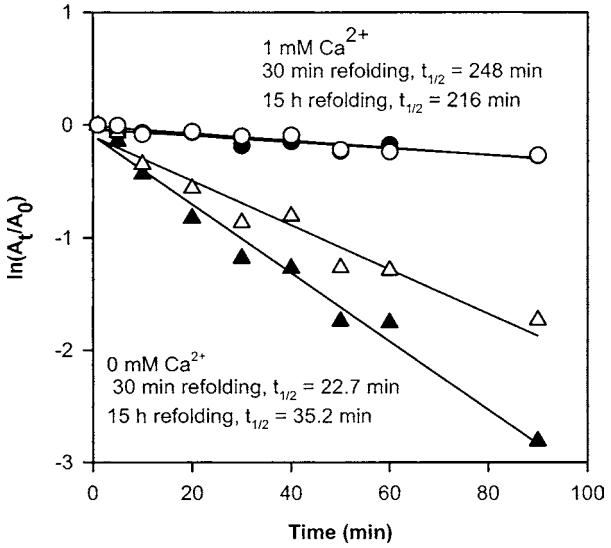

Figure 5. Heat stability curves of CSWP incubated at 75 °C in 25 mM sodium acetate, pH 4.5, with and without 1 mM Ca^{2+}. Samples were removed at the times indicated and incubated at 4 °C for either 30 min or 15 h before residual peroxidase activity was assayed according to the standard assay protocol on ABTS and hydrogen peroxide. Solid symbols, 30 min at 4 °C: (▲) 0 mM Ca^{2+}; (●) 1 mM Ca^{2+}. Open symbols, 15 h at 4 °C: (△) 0 mM Ca^{2+}; (○) 1 mM Ca^{2+}.

pocket (*2*, *3*, *60*). With this in mind, a likely explanation for the protection of CSWP against thermal denaturation by Ca^{2+} is the maintenance of the two calcium ions bound to the peroxidase (presumably by mass action). The protection of CSWP against irreversible thermal denaturation by Ca^{2+} is similar to that found for other plant (*60*, *61*) and fungal (*62*, *63*) peroxidases. Further study of this protection is clearly important and is the subject of future study.

The source of the CSWP in the steep water is unknown but is likely to arise from the corn kernel itself. At least 13 distinct peroxidase isozymes, 3 cationic and 10 anionic isozymes, have been detected in corn by PAGE at pH 8.1 (*64*). These enzymes are widely distributed throughout the plant, including the pericarp, embryo, and endosperm (*64*). Little is known about the properties or structures of the corn peroxidases other than that of an anionic peroxidase, *Zm*AP1, which has been cloned and sequenced (*65*). This enzyme, however, is expressed predominantly in roots, mesocotyl, and coleoptile (*65*). CSWP peroxidase differs significantly from *Zm*AP1, not only in terms of the p*I* but also in terms of the higher lysine levels in the anionic peroxidase (2.76 mol % for *Zm*AP1 vs 1.06 mol % for CSWP).

With few exceptions, most plant peroxidases are glycosylated. The glycans confer a highly hydrophilic surface to the protein and render them less soluble in organic solvents. It has been demonstrated that the removal of the glycans from *Coprinus cinereus* peroxidase by site-directed mutagenesis greatly improves the solubility of the enzyme in acetone (*66*). Attempts in this laboratory to improve the solubility and activity of SBPc in organic solvents by chemically and enzymically removing the *N*-linked glycans, without complete denaturation of the enzyme, have met with limited success (unpublished work). The isolation of a nonglycosylated peroxidase from corn steep water with reasonable thermal and pH stability, particularly under fairly acidic conditions, presents an opportunity to determine if CSWP is more soluble, active, and stable in various organic solvents and to assess its value as an environmentally benign catalyst with industrial potential. Steep water is not a good source for the enzyme because of the large amount of contaminating proteins. However, the degree of purity of the peroxidase will depend on its use, and impure CSWP from steep water may serve well for certain applications. Nonetheless, identification of the source of the enzyme in steep water is clearly of interest in future studies.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BCA, bicinchoninic acid; CM-cellulose, carboxymethyl cellulose; CSW, corn steep water; CSWP, corn steep water peroxidase; HPAEC-PAD, high-pH anion exchange chromatography with pulsed amperometric detection; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MWCO, molecular weight cutoff; PAGE, polyacrylamide gel electrophoresis; RZ, Reinheitszahl [ratio of A_{403} (Soret band)/A_{280}]; SDS, sodium dodecyl sulfate.

ACKNOWLEDGMENT

We thank Blanca Martinez and Bernardo Moreiro for help at the start of the project and Dr. Elena Rus (Molecular Analysis Facility, University of Iowa) for performing the amino acid analyses. We also thank Carrie Olmstead and Dr. Bryce Cunningham (Bio-Research Products, Inc., North Liberty, IA) for performing the flatbed isoelectric focusing.

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Received for review August 9, 2002. Revised manuscript received December 4, 2002. Accepted January 6, 2003. Financial support from the Iowa Biotechnology Byproducts Consortium (USDA Grant 98-34188-5902) is acknowledged.

JF025883N