Stabilization and Partial Purification of a Protease from Ginger Rhizome (*Zingiber offinale* Roscoe)

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ABSTRACT: Ginger protease (GP) or zingibain is of interest as a meat tenderizing agent. The objective of this research was to investigate food-compatible methods for stabilizing GP during storage or enzyme fractionation. Crude GP extracted from fresh ginger had a half-life ($t_{1/2}$) of 2.1 (± 0.16) d at 5 °C decreasing to 20 min at 30 °C. Addition of ascorbate (0.2% w/v) increased the $t_{1/2}$ for GP from 2 to 20 d at 5 °C. Dithiothreitol or Ethylenediaminetetraacetic acid (EDTA) had no effect on GP stability. Acetone powder preparations from ginger yielded GP with $t_{1/2}$ of 18 mo at 5 °C. Crude GP extracted from acetone powder was sufficiently stabilized to allow fractionation by ion exchange chromatography without the addition of toxic or expensive additives. GP was partially purified 252-fold with a recovery of 61%. The nomimal molecular weight of GP was 34.8 kDa compared with 25.1 kDa for papain. This work shows that the stability of GP can be greatly improved, increasing its attractiveness as a commercial product. Some possible routes of GP deactivation and stabilization are discussed.

Keywords: ginger protease, zingibain, stabilization, purification, formulations, ascorbate

Introduction

Ginger is a meat tenderizing agent from antiquity (Pruthi 1980) with both comminuted ginger rhizome or juice being useable. The meat tenderizing component is ginger protease (GP) or zingibain (Thompson 1973; Sakasai and others 1980; Lee and others 1986; Mega and others 1987). GP is highly active against collagen and other connective tissue proteins (Thompson and others 1973). The present market for industrial proteases amounts to about $242 million (US). Two plant proteases (papain and bromelain) account for $17 million (US) sales or 8% market share (Outtrup and Boyce 1990). Ginger is of considerable interest as a potential new source of protease for industry. The low storage stability of GP may limit its use for commercial application.

Zingibain was purified (Ichikawa and others 1973; Ohtsuki and others 1995), crystallized, and the 3-D structure determined by X-ray crystallography (Choi and others 1999; Choi and Laursen 2000). Nevertheless, when this research began, the number of publications dealing with GP numbered less than a dozen. GP is one of the least studied of the plant thiol proteases including papain, bromelain, ficin, and actinidin (Caygill 1979; Storey and Wagner 1986). Isolation techniques for GP have also not been optimized for yield and/or degree of purification. Besides the report by Ichikawa and others (1973), recovery and purification results have not been reported for GP.

The objectives of this research were 2-fold. First stabilization strategies for GP were investigated using 3 enzyme treatments: (1) simple extraction of GP from fresh ginger, (2) addition of chemical additives to GP solutions, and (3) preparation of ginger protease acetone powder (GP_{AC}). Secondly, a method for partially purifying GP in high yield was developed.

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Materials and Methods

Chemicals and materials

Ginger was obtained from a local market. Diethylaminoethylethane (DEAE) Toyopeal-650 ion-exchange chromatography (IEC) support was from Pharmacia-Biotechnology (Uppsala, Sweden). All other chemical were purchased from Sigma-Aldrich, Ltd. (Poole, U.K.). Single distilled water was used in all experiments. Spectrophotometric measurements were using a Cecil model 2002 Instrument (Cecil, U.K.). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) involved a Hoefer SE600 vertical unit (Hoefer Scientific Instruments, San Francisco, Calif., U.S.A.) connected to a Bio-Rad 3000Xi power supply (Bio-Rad, Hercules, Calif., U.S.A.). Chromatography studies used the GradiFrac-100 system with a standard configuration (Pharmacia-LKB, (Uppsala, Sweden). Centrifugation was with a refrigerated Beckman JS2-HS centrifuge (Beckman, Fullerton, Calif., U.S.A.) or an Eppendorf microcentrifuge (Eppendorf model 5415 C, Eppendorf, Germany).

Preparation of ginger protease extract

Freshly diced ginger was homogenized with 4 parts (w/v) cold Tris-HCl buffer (0.05 *M*, pH 8) filtered through 4 layers of muslin and the filtrate centrifuged (12000 × *g*) at 5 °C for 20 min. The supernatant, designated GP was stored at 5 °C. Other GP extracts were prepared similarly with the following buffer additives; dithiothreitol (DTT; 0.3 m*M*), EDTA (1 m*M*), or sodium ascorbate (0% to 1% w/w).

Preparation of ginger acetone powder

Diced ginger was frozen at –30 °C, homogenized with 4 parts cold acetone (–30 °C) for 90 s and allowed to stand for 15 to 20 min. The precipitate formed was collected by vacuum filtration through Whatman nr 1 paper. Suction was continued with further washes with 5 parts (v/w) cold acetone followed by air drying. GP_{AC} was crushed, sieved to remove coarse particles, and stored at 5°C. Enzyme extracts were prepared using GP_{AC} as needed.

Determination of ginger protease specific activity

Enzyme extracts were assayed using azocasein (0.5 mL; 2 mg/mL in 0.05 *M* Tris-HCl buffer, pH 8.0) at 38 °C as described by Sarath and others (1989). The enzyme reaction was stopped by addition of 0.5 mL Trichloroacetic acid (TCA) (30% w/w), followed by micro-centrifugation (12000 × *g* for 10 min) and absorbance measurements at 440 nm. An assay time of 10 to 50 min led to initial rate measurements. The protein content of ginger extracts were determined by the modified Lowry assay (Peterson 1977).

Partial purification of GP from acetone powder

GP_{AC} was prepared as described previously and extracted with 4 volumes of Na phosphate buffer (20 m*M*, pH 7; ± 0.2% w/w sodium ascorbate). The resulting GP_{AC} sample was precipitated with solid ammonium sulphate (70% saturation), centrifuged (12000 × *g*, 5 °C), resuspended in a minimum volume of buffer, and exhaustively dialyzed against Na phosphate buffer.

Dialized GP was fractionated by ion exchange chromatography (IEC) using diethylaminoethyl (DEAE)-Toyopeal 650 column (16 × 450 mm) pre-equilibrated with Na phosphate buffer (20 m*M*, pH 7.0) at 5 °C. First, 20 mL of enzyme (0.6 to 0.8 mg [total protein]/mL) was applied followed by 2 bed volumes of buffer (1 mL/min) and successive applications of 2 bed volumes each of 25 m*M*, 50 m*M*, 100 m*M*, 200 m*M*, and 300 m*M* of NaCl dissolved in Na phosphate buffer. Chromatographic fractions (5 mL) were collected for enzyme activity determination as described previously.

Enzyme molecular weight (MW) determination was using discontinuous SDS-PAGE analysis of Laemmli (1970) with 15% resolving gel and a 4.0% stacking gel and stained with Coomasie Brilliant Blue R-250. In addition, substrate SDS-PAGE was performed as described previously using polyacrylamide gel copolymerized with 0.01% (w/v) bovine serum albumin (Garcia-Carreno and others 1993).

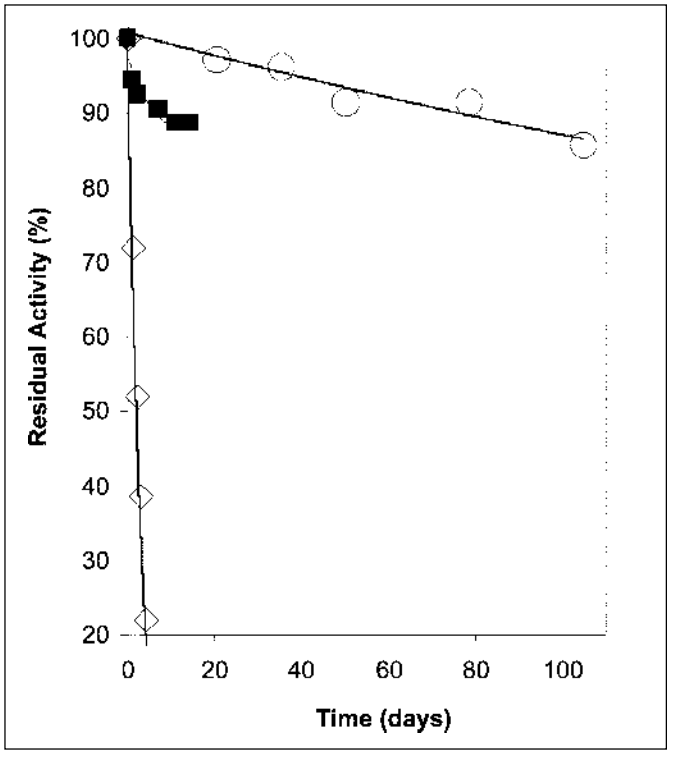

Figure 1—The storage stability of ginger protease (GP) at 5°C. □ = GP; ■ = GP with 0.2% ascorbate, and ○ = GP acetone powder.

Results and Discussion

Stabilization of zingibain

Crude GP extract from ginger was unstable (Figure 1), although the half-life ($t_{1/2}$) is adequate for domestic applications (Thompson and others 1973; Lee and others 1986). In this study, there was 80% loss of activity after crude GP was stored for 4 d at 5 °C. The $t_{1/2}$ value for GP, determined by fitting data to a semi-log plot of ln (activity) versus time, decreased from 2.1 (±0.16) d at 5 °C to 17 min at 30 °C. Because low storage stability is undesirable in a commercial enzyme, 2 stabilization strategies were examined in this study: (1) use of enzyme additives, and (2) acetone powder preparation.

Sodium ascorbate (0.2% w/v) stabilized GP and no activity loss occurred after 14 d at 5°C (Figure 1). Ascorbate also increased GP specific activity by about 5-fold from 1.0×10^{-3} (ΔA_{440}/min/mg) to 5.5×10^{-3} (ΔA_{440}/min/mg). Higher ascorbate concentrations (0.2% to 1.0% w/v) led to no further benefits, and 0.2% ascorbate was used in all further studies. The following compounds had negligible effect on GP stability or specific activity: EDTA (1 m*M*), DTT (0.3 m*M*), and Ca Cl_2 (100 m*M*). The $t_{1/2}$ for GP_{AC} was 18 mo due to the 15% loss of activity after 15 weeks of storage at 5 °C.

Results for thermal stability of GP and GP_{AC} are shown in Fig-

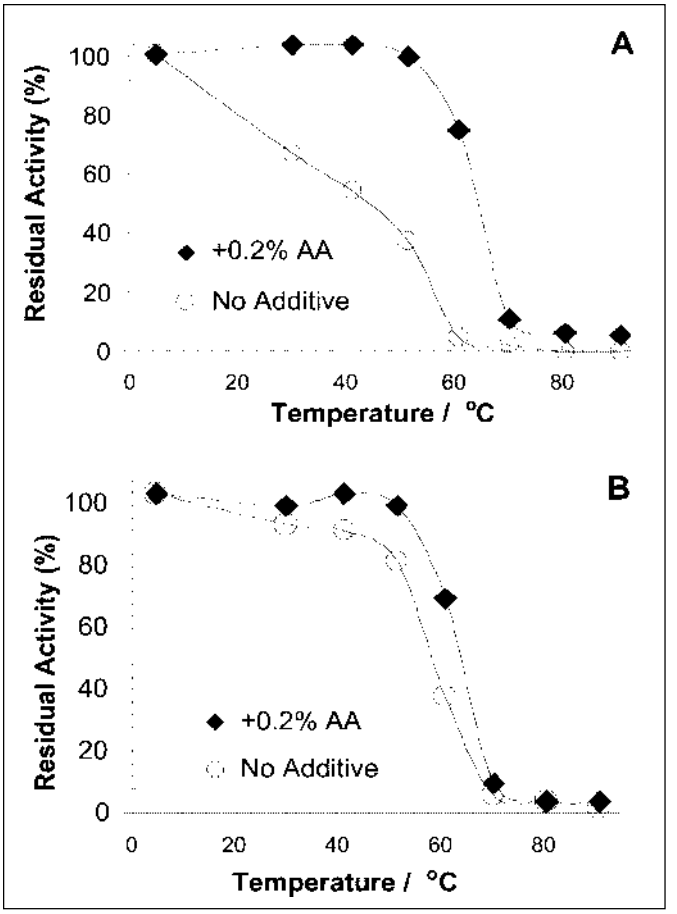

Figure 2—Accelerated testing of the shelf-life stability for crude ginger protease extracts. (a) Stability curves for ginger protease (GP) with or without 0.2% (w/v) ascorbate; (b) enzyme extracts from ginger acetone powder (GP_{AC}) with or without 0.2% (w/v) ascorbate. Enzymes were heated at the indicated temperatures for 10 min, cooled to room temperature, and assayed (see text for details).

ure 2. At 60 °C, the $t_{1/2}$ for GP was 2.3 min or 24 min in the absence or presence of 0.2% (w/v) ascorbic acid, respectively. At temperatures of <50 °C, GP were totally stabilized by ascorbate. In general, GP_{AC} solutions were only modestly stabilized by ascorbate (Figure 2b).

Possible mechanism of zingibain inactivation and stabilization

Improved understanding of GP deactivation may help to design better techniques for enzyme stabilization. Plant thiol proteases require a free SH-group for activity. Some possible routes for GP deactivation are oxidation of the active site SH-group, SH/disulfide exchange, formation of a quinone-thiol adducts, SH-group binding with heavy metal ions, formation of an amino-quinone adduct, and autolysis. GP could also bind to tannins by hydrogen bonding or electrostatic interactions.

Crude GP extracts from fresh ginger underwent rapid browning. The process probably involved the oxidation of polyphenols to form quinones catalyzed by an endogenous polyphenol oxidase (PPO). The quinones could then react with amine, sulfhydryl, and other nucleophilic residues from GP, thereby causing enzyme deactivation. Both DTT and ascorbic acid are inhibitors for browning (Matheis and Whitaker 1983; Jervis and Pierpoint 1989). However, the former did not stabilize GP. Apparently, inhibition of browning does not explain the stabilization of GP by ascorbate.

Autolysis is another well know mode for protease deactivation. Autolysis can usually be avoided by reacting the active site SH-group of GP with cystine or p-chloromercuribenzoic acid (PCMB). The stabilizing effect of ascorbate is not easy to explain in relation to an autolytic deactivation process for GP. The stabilization of GP by ascorbate is thus surprising and somewhat counterintuitive.

Protease stabilization as acetone powder

During acetone powder preparation, hydrophobic plant polyphenols were removed from the ginger. The pulp material also becomes dehydrated by acetone at low temperatures (Caygill 1979). The high stability of GP acetone powder compared with GP from fresh ginger can be explained by the (1) lower concentration of plant pigments and (2) reduced water activity in GP powders. Enzyme 3-D structure is more rigid within low water activity systems (Lui and others 1991; Apenten 1998). Such effects can be readily rationalized by reference to glass transition temperature and the role of water as a plasticizer (Slade and Levine 1991; Champion and others 2000).

Stabilization and purification of ginger protease

Some form of enzyme stabilization is essential during the isolation of GP. Ichikawa and others (1973) stabilized GP with 2 m*M* cysteine. To avoid autolysis, Ohtsuki and others (1995) chemically modified crude GP with PCMB. The mercuribenzoate-GP derivative (MB-GP) was reactivated using DTT or 2-ME before assay.

The GP stabilization strategy adopted in this study was to use GP_{AC} as a stable enzyme source. A buffered GP_{AC} solution (±0.2% ascorbate) was then precipitated with 70% saturated ammonium sulphate, dialyzed, and fractionated by IEC with stepwise gradient elution using NaCl. The total activity recovered by IEC fractionation ranged between 36% (pH 8) and 98.5% (pH 6) without ascorbate as stabilizer (Figure 3a). With addition of 0.2% ascorbate, enzyme recovery ranged between 54% (pH 8) and 118% (pH 7). The >100% recovery is perhaps explained by the removal of an endogenous GP inhibitor during the purification process; moreover, previous results showed that GP was activated when assayed in the presence of ascorbic acid. In summary, GP_{AC} could be successfully fractionated at pH 6 to 7 without added stabilizer.

Degree of purification and yield for ginger protease

Four GP varieties were eluted from the IEC column at pH 7 using 25 m*M*, 50 m*M*, and 75 m*M* NaCl and added ascorbate. The enzyme fractions designated GP_{25}, GP_{50}, and GP_{75} were purified 210-fold, 127-fold, and 102-fold with a recovery of 37%, 14.5%, and 17.6%, respectively. The highest degree of purification (940-fold) occurred with GP_{25} (6.6% recovery) at pH 8.0. GP was also separated into 4 fractions by IEC at pH 7.0 *without added ascorbate* (data not shown). The peaks designated GP_{25}, GP_{50}, GP_{75}, and GP_{100} comprised recoveries of 61.3% (225-fold purification), 14.3%, 19.1%, and 21.1%, respectively, of the total GP applied to the column. The peak activity increase for GP_{25} was 662-fold (10.8% yield).

The MW of GP was 34.8 kDa compared with 25.1 kDa for papain (control). All enzyme fractions had the same MW and produce a single visible band during SDS-PAGE analysis. With substrate-SDS-PAGE the MW for GP was 35.1 to 36.4 kDa compared with 29.2 kDa for papain. The slightly higher MW estimates with substrate-SDS-PAGE is due to enzyme binding with (BSA). The MW for GP was previously reported as 29 kDa (Ichikawa and others 1973; Ohtsuki and others 1995).

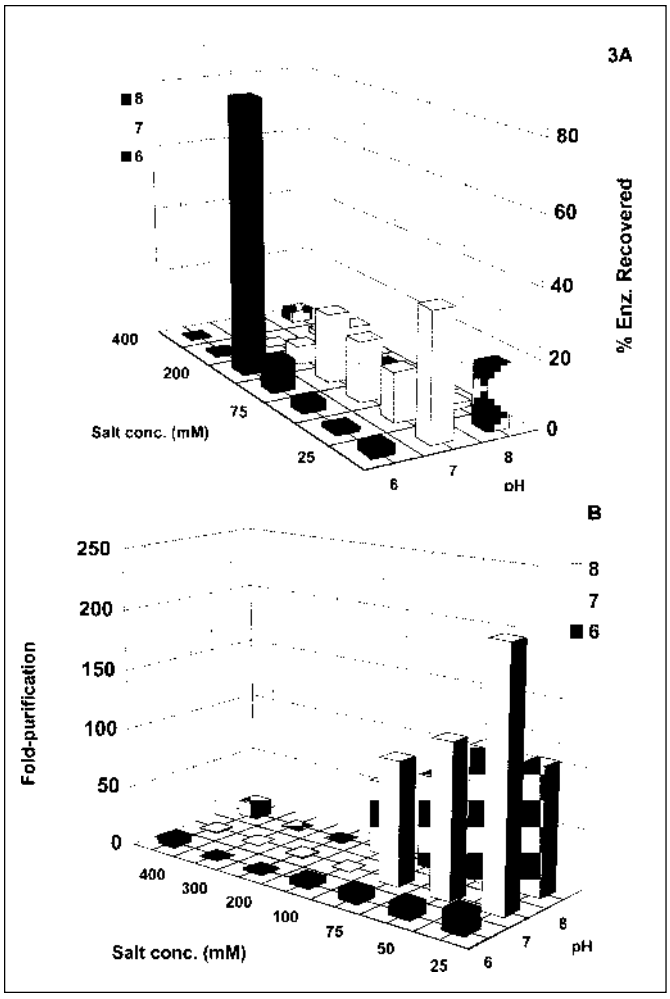

Figure 3—Fractionation of ginger protease by ion-exchange chromatography with stepwise elution using 25 m*M*, 50 m*M*, 77 m*M*, 100 m*M*, 200 m*M*, 300 m*M*, and 400 m*M* NaCl. (a) % total ginger protease (GP) activity recovered; (b) enzyme-purification fold; no stabilizers were used.

Conclusions

Two stabilization methods are described for extending the stor age life for GP for storage and enzyme isolation. Sodium ascorbate stabilized GP solutions by 20-fold at 5 °C. Ginger protease acetone powders were also found to have excellent stability characteristics for storage or enzyme fractionation. Stabilization of GP could make this a more attractive commercial commodity. An efficient method for partially purifying the stabilized GP was described, leading to 940-fold maximum purification.

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