

Notes

Occurrence of trypsin-like protease in cardamom (*Elettaria cardamomum* Maton)

A Josephraj Kumar*, Romit Chakrabarty[§] and
George Thomas[#]

*Cardamom Research Station, Pampadumpara 685 556,
Idukki district, Kerala, India

[§]Department of Plant Biology and Forest Genetics, Genetics
Centre, P.B. No. 7080, Swedish University of Agricultural
Sciences, SE 75007, Uppsala, Sweden

[#]Interfield Laboratories, Kochi 682 005, Kerala, India

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Occurrence of trypsin-like protease in fresh cardamom (*Elettaria cardamomum* Maton) seeds, as evidenced by the benzoyl-arg-*p*-nitroanilide (BAPNA) hydrolyzing ability of the seed enzyme preparation under alkaline condition is reported for the first time. The enzyme has a pH and temperature optima as 8 and 45°C, respectively. It is inhibited by aprotinin and phenylmethyl sulfonyl fluoride (PMSF) in a dose-dependent manner, suggesting the presence of serine residues at the active site. The enzyme had a $V_{\max}$ of 98.01 nmoles *p*-nitroaniline released per min per mg protein and K_m of 0.0684 mM with BAPNA as substrate. Addition of aprotinin (75.75 μ M) increased K_m value by three-folds, whereas the $V_{\max}$ was reduced by 23%.

Keywords: Cardamom, *Elettaria cardamomum* Maton, trypsin, aprotinin.

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Small cardamom (*Elettaria cardamomum* Maton), which is used as a flavouring agent for most culinary and confectionary preparations, is a remunerative spice crop, extensively cultivated in Kerala, Karnataka and Tamil Nadu. More than 95% of cardamom produced in the country is consumed in the domestic market¹. In the Middle-East, cardamom along with coffee is traditionally used in making of a beverage *Gahwa*^{2,3}. It is an essential ingredient of digestive stimulants and especially functions as warming stimulant to digestion^{3,4}. It has been recommended as an aid in digestion especially after heavy meals¹. Furthermore, it is used in medicinal preparations for indigestion and flatulence. In Indian Ayurvedic system of medicine, it is used to remove fats and as a cure for urinary and skin complaints^{1,5}.

Although digestive stimulant-like properties for cardamom have been reported, information on the factors responsible for such effects is lacking. Here, the occurrence of a trypsin-like protease is reported from immature and fresh cardamom seeds for the first time. The enzyme was also partially characterized.

Materials and Methods

Materials

Fresh cardamom capsules were obtained from the research farm of Cardamom Research Station, Pampadumpara, Kerala Agricultural University, Idukki district, Kerala, India. This is one of the hot spot tracts for cardamom cultivation owing to the unique environmental conditions prevailing in the region.

Benzoyl-arg-*p*-nitroanilide (BAPNA), phenylmethyl sulfonyl fluoride (PMSF) and aprotinin were obtained from Sigma-Aldrich (St. Louis, USA). Spectrophotometric measurements were recorded using a Shimadzu UV-VIS 1601 double beam spectrophotometer.

Seed enzyme preparations

Fresh cardamom seeds (2 g) at white seeded stage were homogenized using a pestle and mortar with 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 8.0). Homogenates were clarified to remove particulate matter by centrifugation at 12,000 $\times$ g for 15 min at 4°C. Supernatants containing soluble proteins were transferred to clean tubes, clarified again by the same procedure, and stored at -20°C for use in activity assays.

Enzyme assay

In order to characterize the trypsin-like protease in cardamom, peptidase assay conditions for seeds were standardized for pH range (8-11) and temperature (35-50°C) using a specific substrate for trypsin enzyme benzoyl-arginine-*p*-nitroanilide (BAPNA). Since casein is only a general substrate for all serine proteases, BAPNA was specifically used in our study to determine the occurrence of trypsin-like protease. Peptidase assays were performed in a reaction volume of 300 μ l containing 190-195 μ l of 50 mM Tris-HCl buffer, pH 8 with 20 mM CaCl₂, 5-10 μ l of seed enzyme preparation and 100 μ l of 1 mM BAPNA⁶. The reaction was stopped by the addition of 30 μ l

*Corresponding author

E-mail: entojoe2003@yahoo.co.in

Fax: 04868-236263

acetic acid (30%) after the required period of incubation. Controls were incubated similarly, but acetic acid was added at the beginning of each assay. The peptidase activity was determined by the amount of *p*-nitroaniline (*p*NA) released from the substrate and was measured at 405 nm⁷. It was expressed as nanomoles of *p*NA released/min/g of seed. Total protein in the seed enzyme preparation was determined using bovine serum albumin as standard⁸. Specific activity was represented as activity per mg protein.

Enzyme kinetics

The K_m for BApNA and V_{max} was determined for the seed enzyme preparation in 50 mM Tris-HCl buffer, pH 8 with 20 mM CaCl₂ after incubation for 30 min at 45°C. The enzyme activities were measured using 11 different concentrations of substrate at the range 0.0121-0.4242 mM and the assays were performed in triplicate. The results were analyzed by Lineweaver-Burk plot and values estimated by plotting 1/[s] versus 1/[v]. The K_m and V_{max} were computed from the intercept and slope of the regression equation.

Similarly, aprotinin (75.75 μ M) was added to the reaction mixtures (containing 50 mM Tris-HCl buffer, pH 8 with 20 mM CaCl₂), prior to the addition of 11 different concentrations of substrate at the range 0.0121-0.4242 mM in inhibitor-incubation studies. After derivation of specific activity, the results were subjected to Lineweaver-Burk plot, and using linear double reciprocal plot, K_m for BApNA and V_{max} as inhibited by aprotinin were deduced from the intercept and slope of the regression equation.

Inhibition studies

The effect of trypsin inhibitors, such as aprotinin and PMSF in a range of concentrations 0-166.7 μ M and 0-11.67 mM, respectively was evaluated on the specific activity of seed enzyme preparation *in vitro* with substrate BApNA in pH 8.0 Tris-HCl buffer. PMSF is an irreversible sulphonylating inhibitor of serine proteases and aprotinin has high binding constant to trypsin. Different concentrations of trypsin inhibitors were added into the reaction mixture containing 5-10 μ l of seed enzyme preparation. Other assay conditions remain the same as mentioned above. The peptidase activity was determined by the amount of *p*NA released from the substrate and was measured at 405 nm⁷.

Statistical analysis

Data obtained on the peptidase assay and different concentrations of inhibitors used were subjected to analysis of variance (ANOVA) to determine the significance in pH and temperature optima.

Results and Discussion

The presence of trypsin-like activity as evidenced by the BApNA hydrolyzing ability of the seed enzyme preparation under alkaline condition is reported in cardamom for the first time. Seed enzyme preparation was active over a broad pH range (8-11) and the optimum pH (75.15 nmoles *p*NA released/min/mg protein) was found as 8 ($P < 0.05$; Fig. 1A). Peptidase activity of seed enzyme preparation ranged from 14.80 to 91.82 nmoles *p*NA released/min/mg protein for the various incubation temperatures (35-50°C) studied. Optimum temperature was found to be 45°C, and at higher temperatures the enzyme got rapidly inactivated ($P < 0.05$; Fig. 1B).

PMSF decreased specific activity of seed enzyme preparation from 67.97 to 1.83, whereas aprotinin decreased specific activity from 67.97 to 17.23 nmoles *p*NA released/min/mg protein in a dose-dependent manner ($P < 0.05$; Fig. 1C; Fig. 1D). Micromolar concentrations of aprotinin were effective in the inhibition of seed enzyme preparation. However, in case of PMSF, millimolar concentrations

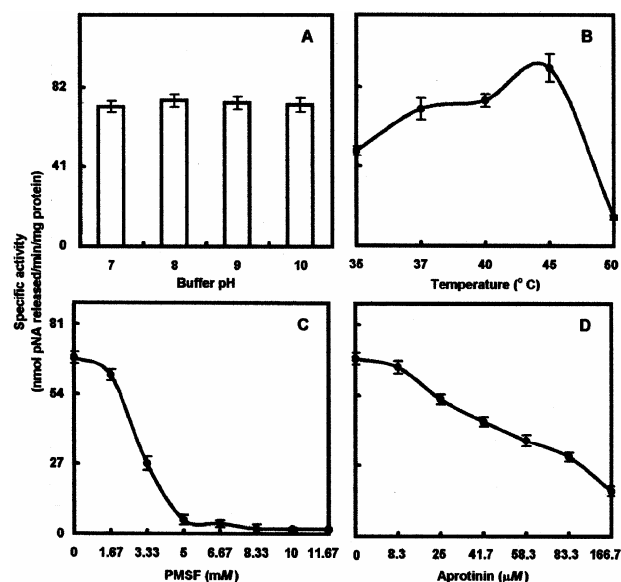


Fig. 1—Influence of buffer composition (A), temperature (B), PMSF (C) and aprotinin (D) on peptidase specific activity of cardamom seed extract [Buffer includes 20 mM Ca plus 50 mM Tris, pH 8.0, except in B, where it varies from 7-10]

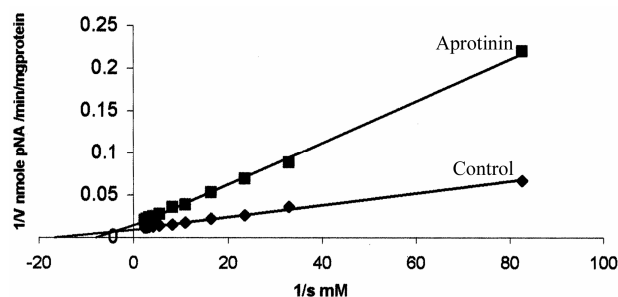


Fig. 2—Lineweaver Burk plot of crude cardamom seed extract with BApNA as substrate and inhibition by aprotinin

were needed to achieve similar levels of inhibition (27 nmoles *pNA* released/min/mg protein). Total inhibition was achieved by PMSF at 5 mM concentration, whereas aprotinin could inhibit 50% specific activity only at 41.7 μ M, suggesting variation in the affinity of two inhibitors, with respect to the concentrations tested on the activity of seed enzyme preparation. We, therefore, suggest that the tertiary structure of aprotinin (a Kunitz-type inhibitor) has the best fit to the active cleft of trypsin-like protease.

The seed enzyme preparation had a $V_{\max}$ of 98.01 nmoles *pNA* released/min/g protein and K_m of 0.0684 mM with BApNA as substrate. K_m for BApNA and $V_{\max}$ as inhibited by 75.75 μ M aprotinin were found to be 0.1871 mM and 75.91 nmoles *pNA* released/min/mg protein, respectively. K_m value as inhibited by aprotinin was 3-folds higher than that of control, whereas the $V_{\max}$ was reduced by 23%. Lineweaver-Burk plot of seed enzyme preparation with BApNA as substrate and inhibition by aprotinin is shown in Fig. 2. A linear double reciprocal plot was obtained with BApNA as substrate. It is evident from Fig. 2 that both K_m and $V_{\max}$ were affected by aprotinin, under the experimental conditions. The data on kinetic parameters suggest that aprotinin is acting similar to un-competitive inhibitor binding to both

free enzyme and enzyme-substrate complex and consequently affecting both K_m and $V_{\max}$.

The BApNA hydrolyzing ability of the seed enzyme preparation under alkaline condition and its inhibition by aprotinin and PMSF, suggest the presence of serine residues at the active site. The BApNA-ase activity of seed enzyme preparation and its inhibition by trypsin inhibitors confirm the presence of trypsin-like protease activity of cardamom, which could possibly be responsible for enhanced digestion and reduction of abdominal gases⁵, as trypsin plays a pivotal role in digestion, and trypsinogen accounts for 20% of the protein in pancreatic fluid⁴.

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