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Title: Purification, Characterization and Crystallization of Protein Protease Inhibitor from Plant Source

ABSTRACT

In this study, ASPI was purified from garlic bulbs. The purity and molecular weight of ASPI was determined by SDS PAGE analysis where a single thick band corresponding to a molecular weight of approximately ~15 kDa indicated the output with high level of purity. The band was further destained and trypsin digested for MALDI-TOF-TOF MS analysis. These fragments were found to be the part of Truncated Trypsin Inhibitor (gi|13375351) which depicted a clear picture confirming the protein enzyme as Trypsin Inhibitor. Catalytic properties of purified ASPI showed that the optimum temperature of purified ASPI was 30°C. It was observed to be most active only up to 50°C recording a maximal inhibition in the range of 30–70°C. After 70°C, there was an eventual drop in the activity due to loss of structure and its aggregation. Purified ASPI showed optimum activity at pH 8.2 and stability at pH 8.0 using BAPNA as substrate. The results obtained suggest that ASPI was stable in neutral to mild alkaline and acidic range with maximum activity at pH 8.2. There was a drop in activity and aggregation of protein at pH 4.5 which happens to be its pI value. The results depicted that ionic and nonionic detergents except SDS have negative effect on ASPI activities. There was an increase in residual activity of ASPI to ~143% in the presence of 1% SDS as compared to control. In case of Tween 80 and Triton X-100, the residual inhibitory

activities decreased with increasing the concentration of detergents used. Oxidizing agents such as H_2O_2 and DMSO reduced ASPI activity in concentration dependent manner. The results showed that 5mM H_2O_2 almost completely inhibited ASPI activity whereas 5mM DMSO reduced ASPI activity to ~21% suggesting that oxidation can be a key element for ASPI activity regulation. Moreover, reducing agent like DTT also reduced the residual inhibitory activity of ASPI in concentration dependent manner. The activity was reduced to 8% at 5mM DTT concentration. Whereas, β-ME could not inhibit ASPI activity to an appreciable level showing maximum drop to ~85% at 5mM β-ME. The kinetic analysis revealed sigmoidal relationship of velocity with substrate concentration with V_{max} 240.8 (μM/min) and K_m value of 0.12 μM. The low Km value suggests higher affinity of enzyme (trypsin) towards substrate (BAPNA). In the presence of ASPI, there was a decline in V_{max} value and the curves intersecting each other on X axis at $-1/K_m$. The mode of trypsin inhibition by ASPI was non-competitive. The K_i value calculated from Dixon plot was 0.08±0.01 nM). The biophysical analysis of purified ASPI depicted 2.0% α -helices and 51% β -sheets at native pH. The reversibility of protein has been confirmed both by thermal and urea induced denaturation which demonstrates two-state denaturation of ASPI. The studies depict denaturation is reversible and follows a two-state mechanism. The biochemical analysis of ASPI was performed by determining the activity against *Aedes Aegypti* by inhibiting its midgut proteases obtained from 3rd stage instar larva with The IC_{50} value of 50.566±0.33 μg/ml. ASPI also caused delayed larval development and mortality. Acute toxicity of ASPI on 3rd instar *Ae. aegypti* larvae was dose-dependent, with an LC_{50} value of 431.91 ug/ml. For an initial screen of crystallization, the crystals obtained could not be diffracted due to various conditions but were observed under the microscope. They showed up either rectangular or rod like structure grouped in form of clusters which was quite similar to the orthorhombic crystals of PI from *Tamarindus indica.*