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Full Length Research Paper

Kinetics studies of protease in fermenting locust beans (*Parkia biglobosa*) and melon seed (*Citrullus vulgaris*)

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Melon seed (*Citrullus vulgaris*) and locust bean (*Parkia biglobosa*) were separately subjected to a natural solid – substrate fermentation for 5 days. The peak of protease (P.I) production from the fermenting melon seeds was 48 h while that of the protease (P.II) from fermenting locust bean was 96 h. Both P.I and P.II demonstrated maximum activity at 40°C. The peak of activity for P.I was observed between pH 6.6 to 7.6, whereas for P.II, the optimum pH was 5.8. The Michaelis – Menten's constant (K_M) as well as the maximum velocity (V_{Max}) of activity were 4.8 x 10^{-2} M and 3.43 x 10^{-2}/s, and 5.8 x 10^{-2} M and 5.1x10^{-2}/s for both P.I and P.II respectively. The work concludes that fermenting melon and locust bean seeds may be a cheap and alternative source of proteases for industrial processes.

Key words: Kinetics, protease, natural fermentation, locust bean, melon seed, alternative sources.

INTRODUCTION

Proteases are a group of enzymes that catalyze the cleavage of peptide bonds in proteins, thereby causing the disassembly of protein molecules. They differ in their ability to hydrolyze various peptide bonds as each type of protease has a specific kind of peptide bond it cleaves.

They can be obtained from animal, plant and microbial sources some examples include, trypsin, chymotrypsin (animal pancrease), pepsin (gastric stomach), bormelain (pineapple), papain (papaya latex), ficin (fiscus latex), substilisin (*Bacillus subtilis* collagenase (clostridia), fungal proteases, viral proteases and other bacteria proteases are also kwon (Rao et al., 1998).

Proteases execute a large variety of complex physiological functions; their importance in conducting essential metabolic and regulatory functions as evident from their occurrence in all forms of living organisms. Some of the functions includes activation of zymogens and blood coagulation (Shimomura et al., 1995), cell growth and migration (Liaw et al., 1999), morphogenesis, tissue arrangement (Zahedi, 2001) inflammation (Holzhausen et al., 2005). Proteases also catalyse important proteolytic steps in tumor invasions or infection cycle of a number of pathogenic micro-organism and vessels, a 'quality' that makes proteases a valuable target for new pharmaceuticals, they also aid metastasis (Camera et al., 2004). Pozo et al. (2004) reported that proteases can be involveed in fungal development and have been related to pathogenic or bio-control of pests and diseases in stored grains where fungal infections are major problems.

Besides the numerous physiological functions of proteases they have a variety of applications in the food and detergents industry (Phadatare et al., 1993). Fungal neutral proteases supplement the action of plant, animal and bacterial proteases in reducing bitterness of food protein hydrolysate. Fungal alkaline proteases are also used in food protein modification (Impoolsup et al., 1981). In view of the recent trend of developing environmentally friendly technologies, proteases are seen to have extensive application in leather treatment (tanning) (Kamini et al., 2004) and in several bioremediation processes and in pharmaceutical industries for debridement of wounds (Roa et al., 1998).

The inability of plant and animal proteases to meet current world demand has led to an increased interest in microbial proteases both for basic understanding of enzyme mechanism and industrial applications (Rao et al., 1998). Microorganisms represent an excellence source of enzymes owing to their broad biochemical diversity, limited spaces required for their cultivation and their susceptibility to genetic manipulation. Protreases are ubiquitous

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in microorganisms and except for a few carboxypeptidases, the majority of these enzymes are endopeptidases (or proteinases) which are predominantly extracellular and are isolated in active forms from the culture filtrates of the appropriate organism (Matsubara and Feder, 1971). Some of these organisms are natural associates of Nigerian fermented foods. For instance, *B. subtilis* and *Aspergillus spp* (Odunfa, 1981) are common isolates of dadawa production. However dadawa production can be from locust beans (Achi, 1992) or from melon seed (Jideani and Okeke, 1991) depending on the locality. Kuddus et al. (2005) have shown that *Bacillus spp* and *Staphylococcus spp* are bacteria isolates associated with fermenting foods. Such extracellular proteases from fermenting foods, particularly from the production of dadawa, a popular food seasoning in Nigeria, have not been studied. It is on this wise that the present work is designed to study the characteristics of extracellular proteases produced in the process of fermenting locust beans and melon seeds.

MATERIALS AND METHODS

Source of material and chemicals

African locust bean and melon seeds were obtained from the local market in Bida, Niger State, Nigeria, while most of the chemicals used were of the Analar grade from BDH Chemicals Limited. Poole, England.

Fermentation of seeds

The fermentation of locust bean and melon seeds was carried out according to the method reviewed by Achi, 2005.

Production of dadawa

Raw African locust bean was boiled for 12 h and further soaked in the boiling water another 12 h (preferably overnight). Excess water was drained off and the seeds were dehulled by marching in a large wooden mortar and further removal of the seed coat was achieved by rubbing the cotyledons between the palms of the hand. The cotyledons were again cooked for another 6 h, drained off and the cotyledons were then spread into trays, covered with jute sacks and fermented for 5 days to produce dadawa

Crude enzyme extraction

Locust bean and melon seeds (500 g each) were allowed to ferment as described above and samples were taken from the fermenting seeds at 24 h interval 5 days. Samples (1 g) collected in each case were suspended in 5 ml of pre-chilled 0.02 M phosphate buffer (pH 7.0) and the mixture was blended in warren blender and stirred, the resulting homogenate was centrifuged at 10000 X g for 10 min. The resulting supernatant constitutes the crude enzyme extract which was used for subsequent analysis. Proteases from fermenting locust bean and melon seeds are designated as P_1 and P_{11} respectively.

Assay for protease activity

Proteolytic activity was determined by monitoring the rate degradation of protein (1% albumin) by a modification of the biurette method according to Henry et al. (1974). 1 unit of enzyme activity was defined as the amount of enzyme required to degrade a unit of albumin (mMol/s) in the reaction mixture condition.

Proteases activity levels were monitored at 24 h intervals as fermentation progressed from 0 - 120 h, enzyme for subsequent analysis was harvested at the highest enzyme yield.

Determination of kinetic parameters

The Michaelis-Menten's constant (K_M) and the maximum attainable velocity (V_{Max}) were determined by investigating the effect of substrate concentration on enzyme activity. Enzyme activity was determined at different substrate concentrations [s], 0.1 – 1 mg/ml, the Lineweaver-Burk plot (1/V vs 1/[S], where V is the reaction velocity) was then constructed, and from this graph, the K_M and V_{Max} were determined for both P_I and P_{II}.

Effect of temperature on proteases activity

Reaction mixture was incubated at different temperatures (T) viz: 15, 20, 30, 35, 40, 45, 50 and 55^0C and the optimum temperature was determined from the graph of enzyme activity Vs temperature (T).

Effect of pH on proteases activity

Proteases activity was determination at various pH values, 0.02 M phosphate buffer at pH values ranging between 5.0 and 7.8 were used to prepare crude enzyme extract as earlier described and the proteases activity of each enzyme extract was assayed. From the result obtained a graph of enzyme activity Vs pH was plotted, and the pH optima for P_I and P_{II} were subsequently determined.
The results are mean of three determinations.

RESULT AND DISCUSSION

Fermentation

Protease activity profile with fermentation time is shown in Figure 1 and reflects that maximum yield of P_I is at 48 h, while P_{II} production was highest at 96hrs. P_I activity increased rapidly between 24 and 48 h and began to decrease afterwards, while for P_{II}, activity increased rapidly between 48 and 98 h and declined progressively afterwards. This observation suggests a period of maximum growth of fermenting organisms and protease yield in fermenting melon seeds and locust bean within 2 days and 4 days, respectively. This agrees with the report of Jidean et al. (1989), who reported that good aroma development is enhanced between 2 – 5 days of fermenting locust bean and melon seed at 37 and 40°C

Optimal temperature and pH

Temperature and pH-activity profiles of protease from fermenting locust bean and melon seed are shoen in Figures 2 and 3. The result (Figure 2) of the effect of temperature on the proleolytic activity on albumin (1%) indicated that both P_I and P_{II} demonstrated maximum activity at 40°C under the assay conditions. The same range of

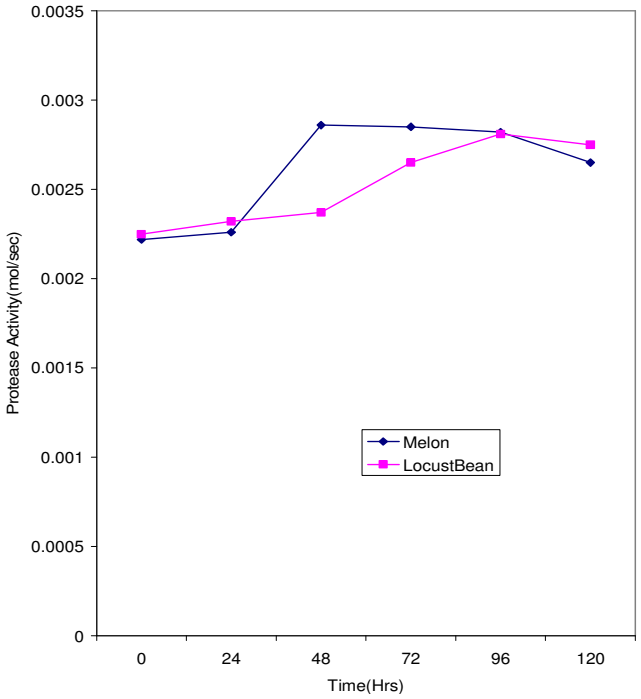

Figure 1. Protease activity profile with fermentation time.

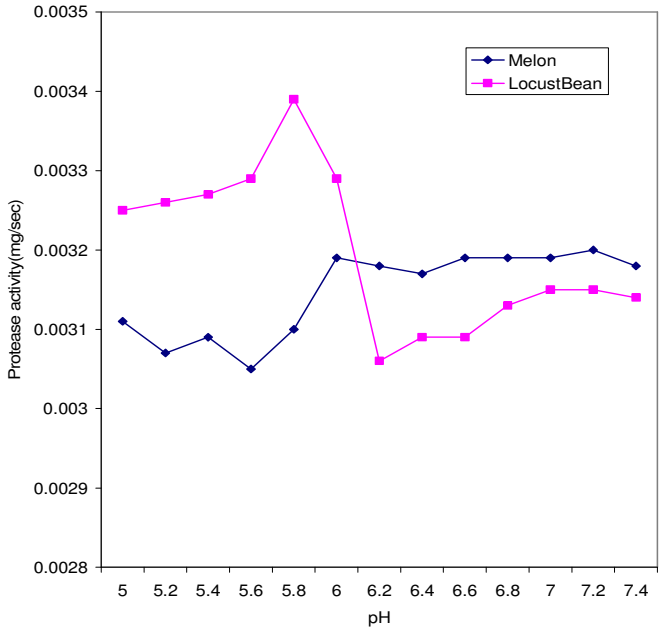

Figure 3. pH activity profile of fermenting melon and locust bean seed.

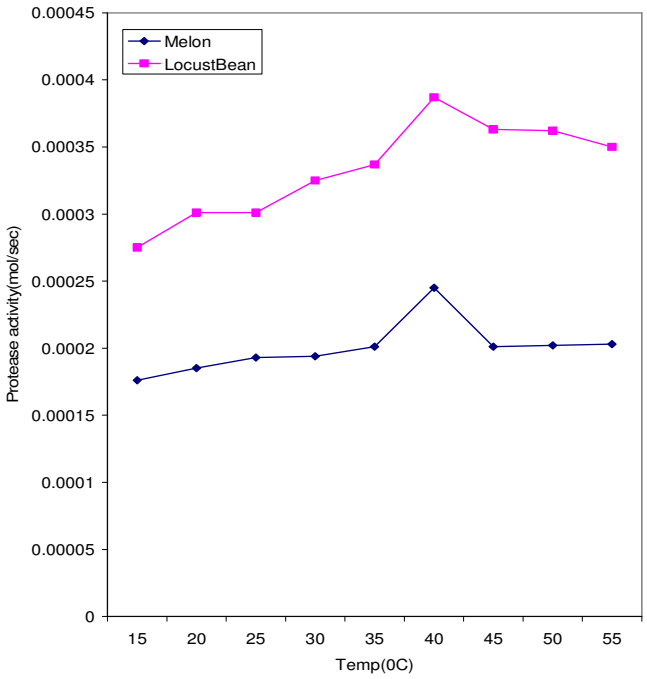

Figure 2. Temperature protease activity in fermenting melon and locust beans seed

temperature has been reported for protease activity isolated from *Bacillus lecheniformis* (Al-sheri and Mustafa, 2004). This result also suggests that protease from fermenting melon seeds and locust bean may be applied in industrial processes with mild temperature as in leather industry (Kamini et al., 2004).

Crude protease from fermenting locust bean and melon seed showed maximum activity at pH 5.8 and 6 - 7.2 respectively (Figure 3). The fact that the activity of protease from fermenting melon was almost constant between pH 6.6 and 7.6 and that a single peak of activity at pH 5.8 was observed for crude protease from fermenting locust bean is an indication that a single acid protease and neutral were involved in each case. Though crude protease from fermenting melon seeds exhibited a relatively broad pH optima, it is suspected that different groups of peptide bonds were possibly hydrolysed in the substrate, moreover, the rate of cleavage of any particular susceptible bond is dependent upon the charge on the substrate molecule and hence, on the pH of the medium (Martin and Jonsson, 1965). That the crude protease from fermenting melon seeds and locust bean shared the same temperature optimum and different pH optima indicates that these protease could have different industrial applications or may be applied to different stages of an industrial process. This agrees with the report of Kuddus et al., (2005), who have shown that protease isolated from different organisms having different temperature and pH optima could have different industrial and biotechnological applications.

Enzyme kinetics

On examining the effect of substrate concentration on proteolytic activity, crude protease from fermenting melon seed and locust bean were found to obey the Michaelis-

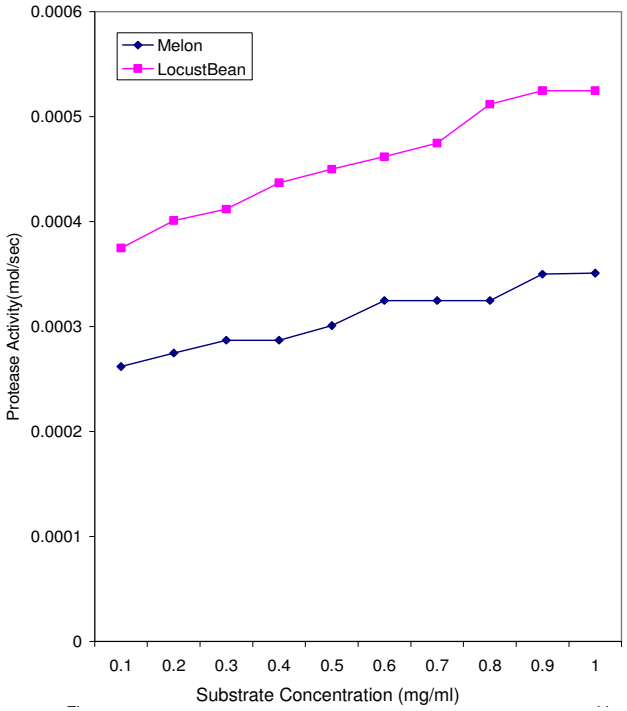

Figure 4. Substrate activity profile of protease from fermenting melon and locust bean seed.

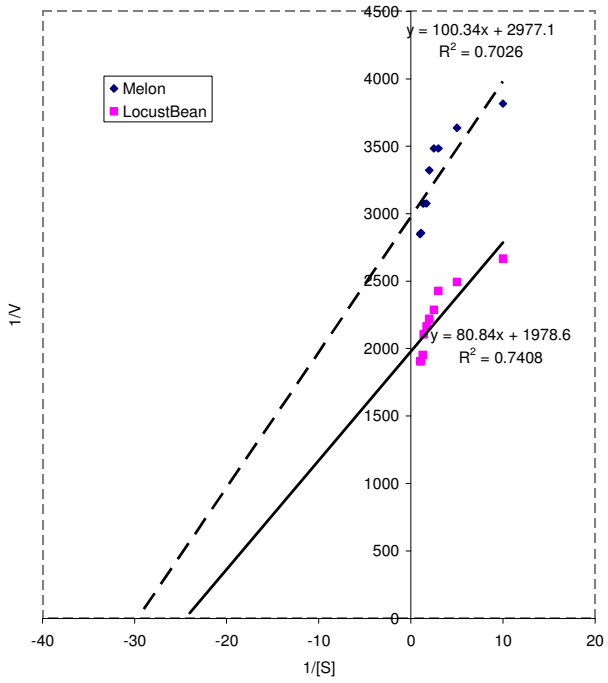

Figure 5. Line weaver -burk plot of protease from fermenting melon and locust bean seed.

Menten kinetics (Figure 4).

From the Linweaver-burk plot (Figure 5), the K_M of crude protease from fermenting locust bean and melon seed were 5.8 X 10^{-2} M and 4.8 X 10^{-2} M while the V_{Max} were 5.1 X 10^{-2} s^{-1} and 3.43 X 10^{-2} s^{-1} respectively, this shows that the enzymes exhibited considerable proteolytic activity on albumin.

However, protease from fermenting locust bean has a higher affinity for the substrate and higher proteolytic activity than protease from melon seed. This therefore suggest that crude protease from fermenting locust bean (P_1) may have more industrial applications particularly in egg processing.

The present work concludes that fermenting locust bean and melon seed may be an alternative and cheap source of industrial protease. However, further work may be required to find the activity of the crude and pure enzymes on other substrates.

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