

Partial Characterization of Protease from the Leaves of *Jatropha curcas*

M. A. Ibrahim¹, J. O. Olatominwa¹, A. B. Aliyu², M. Bashir¹ & A. B. Sallau¹

¹ Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

² Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria

Correspondence: M. A. Ibrahim, Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria. Tel: 234-703-110-4932. E mail: mauwalibrahim@gmail.com, maibrahim@abu.edu.ng

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Abstract

The crucial roles of proteases in food and other industries stimulate research to find additional sources of the enzyme especially from non-conventional sources. In this study, protease was partially characterized from *Jatropha curcas* leaves. The enzyme had pH and temperature optima of 4.0 and 45°C respectively. A decline in residual activity of the enzyme was observed at above 45°C and the activation energy (E_a) from Arrhenius plot was 0.57kJ/mol. The enzyme showed specificity in the order; casein>hemoglobin> albumin> ovalbumin. Initial velocity studies for the determination of kinetic parameters revealed a K_M and V_{max} of 0.48 mg/ml and 0.014 μ mol/min respectively with a computed index of physiological efficiency (K_{cat}) of 0.029 min⁻¹. Furthermore, Dixon-Webb's plot identified ionizable groups at the active site with pKa₁ and pKa₂ of 5.0 and 5.3 respectively as well as an enthalpy of ionization of 0.047 kcal/mol implicating aspartate as an important amino acid at the active site of the enzyme. The protease was highly sensitive to cysteine protease specific inhibitor iodoacetate while 1,10phenanthroline and ethylenediaminetetraacetate slightly inhibited the enzyme. Data from this study suggest that *J. curcas* protease possesses closely similar properties to other known industrial proteases.

Keywords: enzyme kinetics, industrial process, *Jatropha curcas*, protease

1. Introduction

Enzymes have evolved to become an integral part of many industrial processes and among the different classes of enzymes, proteases constitute 60% of the total worldwide sale of enzymes used in various industries and also account for at least a quarter of the total global enzyme production (Gupta, Beg, & Lorenz, 2002). Proteases have important industrial applications which include detergent, food, leather and meat tenderization industries (Cheng, Lu, Li, Liu, & Liang, 2010). They are also important tools in studying the structure of proteins and peptides. Furthermore, these enzymes are used in pharmaceuticals, medical diagnosis, and decomposition of gelatin on X-ray films as well as in textiles (Tunga, Shrivastava, & Banerjee, 2003). Although most available reports on proteases are derived from microbial origin (Tang et al., 2003; Soares, Castilho, Bon, & Freire, 2004; Ire, Okolo, & Moneke, 2011) but commercial use of proteases with different origins for hydrolysis of proteins was reported to be very promising due to the biological origin of enzymes (Capiralla, Hiroi, Hirokawa, & Maeda, 2002). Plant cysteine proteases such as papain and stem bromelain are still extensively used for medicine, brewing wine, and food industry (Kaneda, Yonezawa, & Uchikoba, 1997). Thus, plant proteases with important industrial applications have also been characterized (Fahmy, Ali, & Mohamed, 2004; Yang, Song, Gu, & Li, 2011).

Jatropha curcas is an arid plant belonging to the family Euphorbiaceae and is common in most arid areas of Asia, South America and Africa. The plant is a common shrub of 3–6 m and the leaves are medicinally used as remedy against rheumatism and jaundice. The seeds are rich sources of oil and the oil obtained from the seeds is used up in the soap industry and also as source of energy (Subramani, Chandrashekharaiyah, Swamy, & Murthy, 2012). The plant has attained economical importance due to these applications in areas with extreme climates and soil conditions because of its extraordinary high drought resistance (Gubitz, Lischnig, Stebbing, & Saddler, 1997). However, in many parts of the world including ours, the plant is still merely used as live fence and for erosion control. In order to exploit more commercial potentials of this plant and search for more sources of plant proteases, the present work was undertaken to partially characterize protease from the leaves of the plant with a

view to obtain preliminary kinetic data for the enzyme which could be useful in assessing its overall suitability in industrial processes.

2. Materials and Methods

2.1 Plant Material

Jatropha curcas fresh leaves was collected from Zango village, Zaria, Kaduna state, Nigeria and was identified to the species level at the herbarium unit of the Biological Sciences Department, Ahmadu Bello University Zaria-Nigeria where a voucher specimen (No. 1911) was deposited. The leaves were thoroughly washed with five changes of distilled water and refrigerated at 4°C until needed.

2.2 Crude Enzyme Extraction

The crude enzyme was extracted according to Li, Cao, Gu and Wen (2011) with slight modifications. Briefly, 10 g of the leaves was homogenized with cold phosphate buffer pH 6.8 (3 x 30 ml). The homogenate was then filtered with a cheese cloth and centrifuged at 5000 xg. The supernatant was collected and kept on ice while the pellet was resuspended in 30 ml of the extraction buffer. This homogenate was similarly treated and the supernatant collected. The combined supernatants were used for the protease activity assay.

2.3 Protease Assay

The protease activity was assayed as described by Fahmy et al. (2004). Briefly, 50 µl of the crude enzyme source was incubated with 500 µl of 100 mM sodium acetate buffer pH 4.5, and 100 µl of 3% casein at 37°C. The mixture was then made up to 1ml with distilled water. Assays were carried out after 1 h, the reaction was stopped by the addition of 200 µl of 20% trichloroacetic acid. The precipitated proteins were removed by centrifugation at 10,000 xg and the absorbance of the supernatant was measured at 366 nm. The activity of protease was defined as the amount of enzyme that hydrolyzes 1µmol of amino acids (in terms of tyrosine) from casein per minute under the standard assay conditions.

2.4 pH and Temperature-dependent Studies

A pH dependent assay of the enzyme was conducted using 50 mM acetate buffer pH 3.0-5.0; 50 mM phosphate buffer pH 6.0-7.0 and 0.1 M TrisHCl buffer pH 8.0-9.0. The temperature-dependent study was performed by determining the enzyme activity at varying temperatures ranging between 20 and 60°C. In order to study the thermostability of the enzyme, the enzyme was preincubated at various temperature (20-60°C) for 1 h and thereafter the residual activity was assayed. An Arrhenius plot of log of enzyme activity (Log V) versus reciprocal of absolute temperature (K^{-1}) was constructed to determine the activation energy (E_a) of the enzyme.

2.5 Substrate Specificity and Kinetic Studies

Substrate specificity studies were carried out using the following substrates; albumin, hemoglobin, casein and ovalbumin. Kinetic experiments were performed using varying concentrations of casein (0.94-30 mg/ml) and the initial velocity values obtained were used to determine the K_M , V_{max} and hence the computed index of physiological efficiency (K_{cat}) of the *J. curcas* protease from the double reciprocal plot of the data. Furthermore, initial velocity data at varying pH values (3.0-9.0) was used to construct a Dixon-Webb's plot of log of V_{max}/K_M against the corresponding pH values to determine the possible ionizable groups in the active site of the *J. curcas* protease.

2.6 Effect of Some Divalent Cations and Some Inhibitors on *J. curcas* Protease Activity

The enzyme assay described above was performed in the presence of chloride salts of the following divalent ions; Ca^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+} and Cu^{2+} at 10 mM final concentrations as well as 0.05 M iodoacetate (IAA), 0.05 M 1,10 phenanthroline (PT) and 0.05 M ethylenediaminetetraacetate (EDTA).

3. Results

The pH dependent profile of the *J. curcas* protease revealed an optimum activity at pH 4.0 and relatively lower enzyme activity was observed at alkaline pH in comparison to acidic pH values (Figure 1). The temperature dependent studies showed a typical bell shaped curve with optimum activity at 45°C (Figure 2). A sharp decline in residual activity of the *J. curcas* protease was observed at above 45°C in a heat stability studies (Figure 3) and the Arrhenius plot gave an E_a value of 0.57 kJ/mol for the *J. curcas* protease (Figure 4).

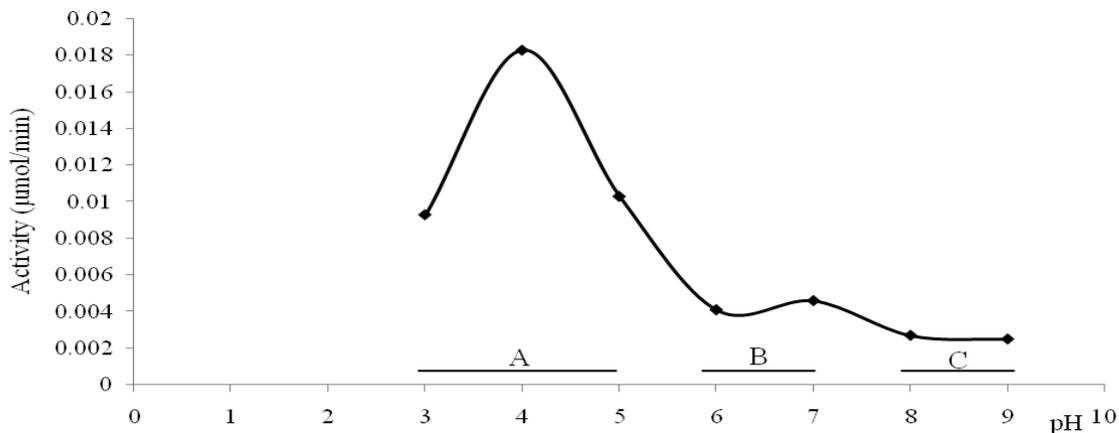


Figure 1. Effect of pH on the activity of protease from *J. curcas* protease. The type of buffer used in the region A, B and C are 50 mM acetate buffer, 50 mM phosphate buffer and 0.1M TrisHcl buffer respectively

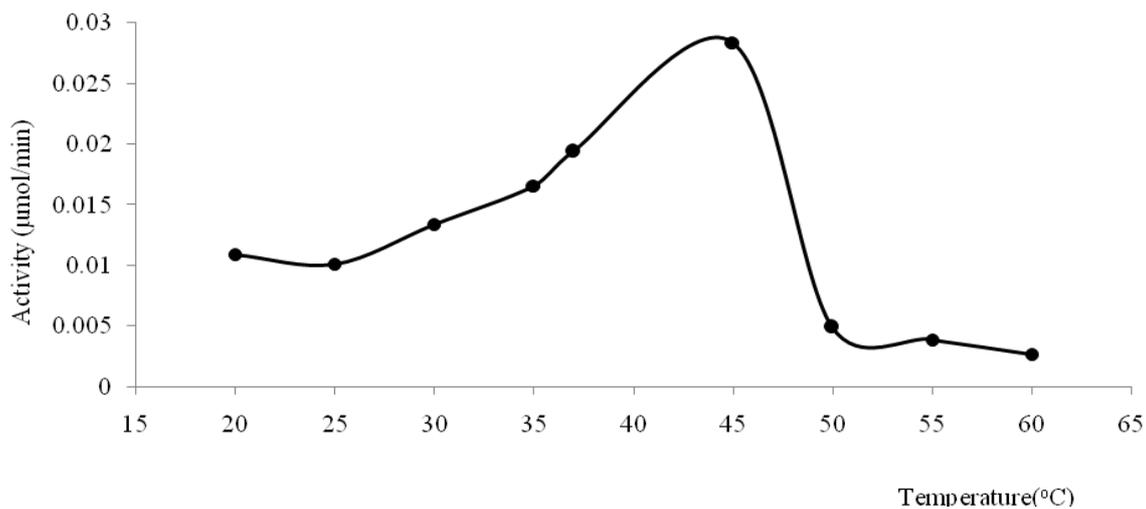


Figure 2. Effect of temperature on the activity of *J. curcas* protease

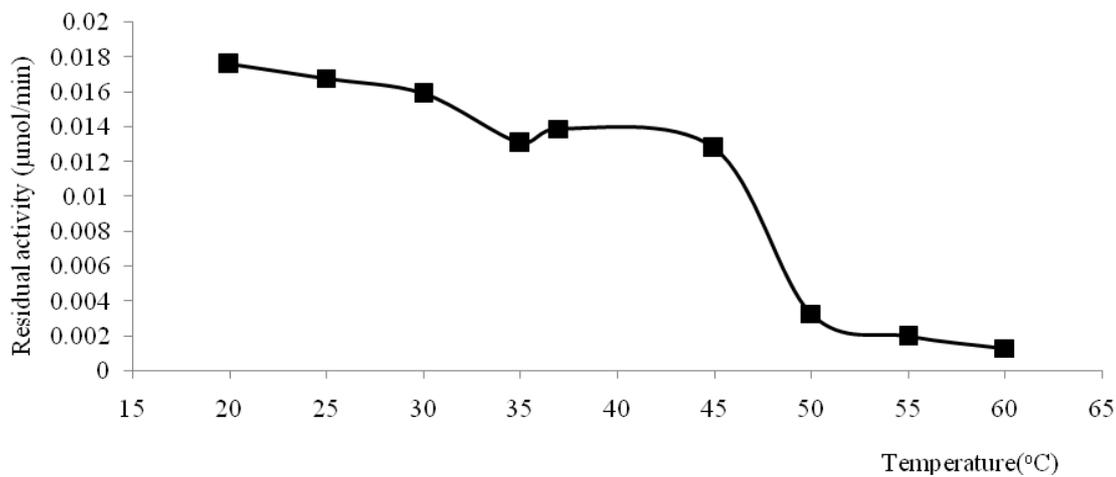


Figure 3. Thermostability profile of protease from *J. curcas* leaves

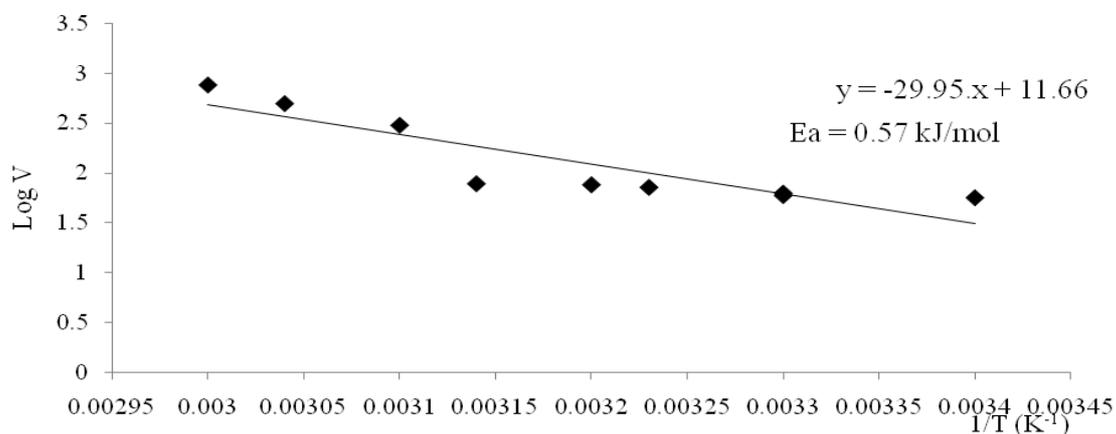


Figure 4. Arrhenius plot for the determination of activation energy for *J. curcas* protease. Each point represents the average of three experiments

The enzyme showed specificity in the order; casein > hemoglobin > albumin > ovalbumin. Steady state kinetic analysis from the initial velocity studies using casein as substrate revealed a K_M and V_{max} of 0.48 mg/ml and 0.014 $\mu\text{mol}/\text{min}$ respectively (Figure 5) with a K_{cat} of 0.029 min^{-1} . Dixon-Webb's plot (Figure 6) for the determination of ionizable groups in the active site of the enzyme gave a pK_{a1} and pK_{a2} of 5.0 and 5.3 respectively with a calculated enthalpy of ionization of 0.047 kcal/mol.

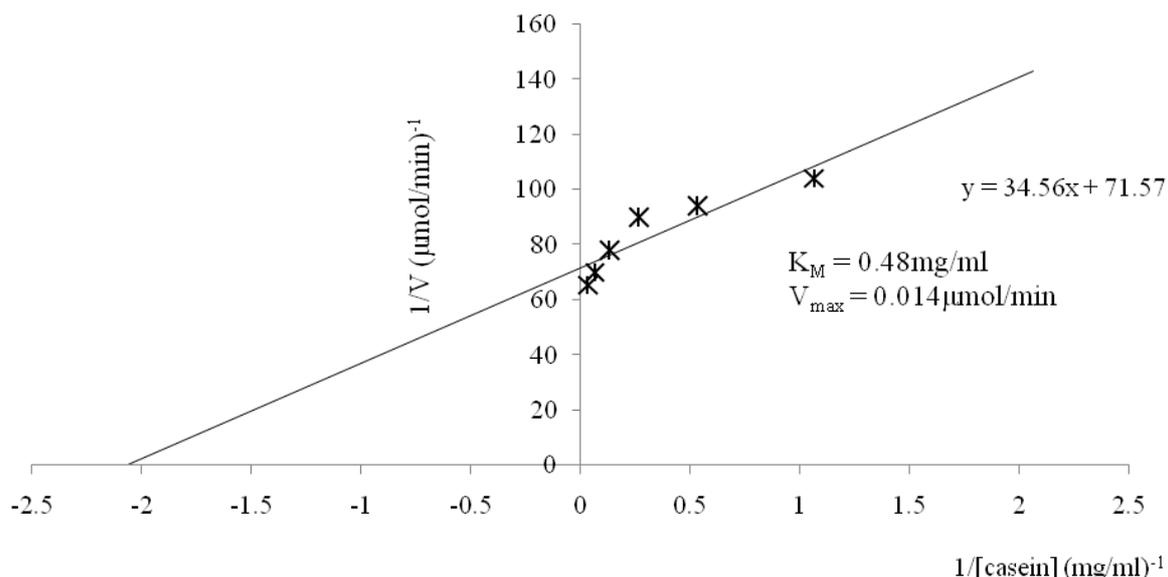


Figure 5. Lineweaver-Burke plot relating initial velocity data of the *J. curcas* protease with casein concentration. Each point represents an average of three experiments

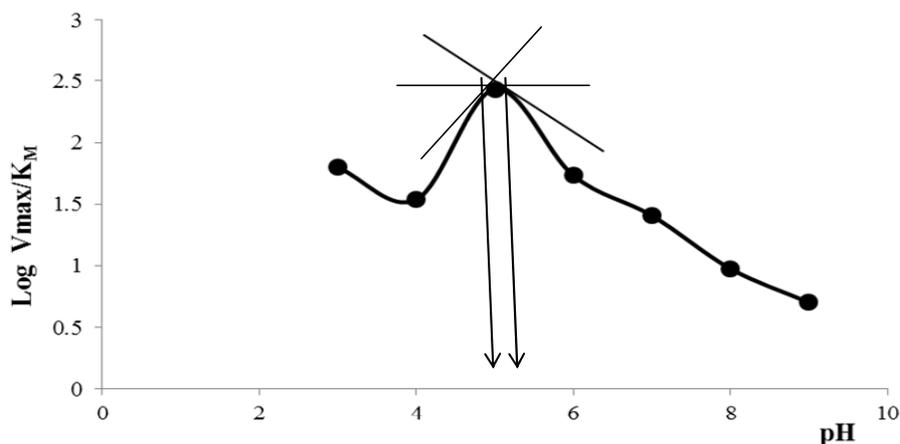


Figure 6. Dixon-Webb's plot for the determination of possible ionizable groups in the active site of *J. curcas* protease

Of the divalent cations tested on the protease, only Ca^{2+} , Mg^{2+} and Fe^{2+} increased the activity whereas Co^{2+} , Zn^{2+} and Cu^{2+} were inhibitory to the enzyme. The *J. curcas* protease was highly sensitive to a typical cysteine protease inhibitor, IAA while PT and EDTA were slightly inhibitory to the enzyme (Figure 7).

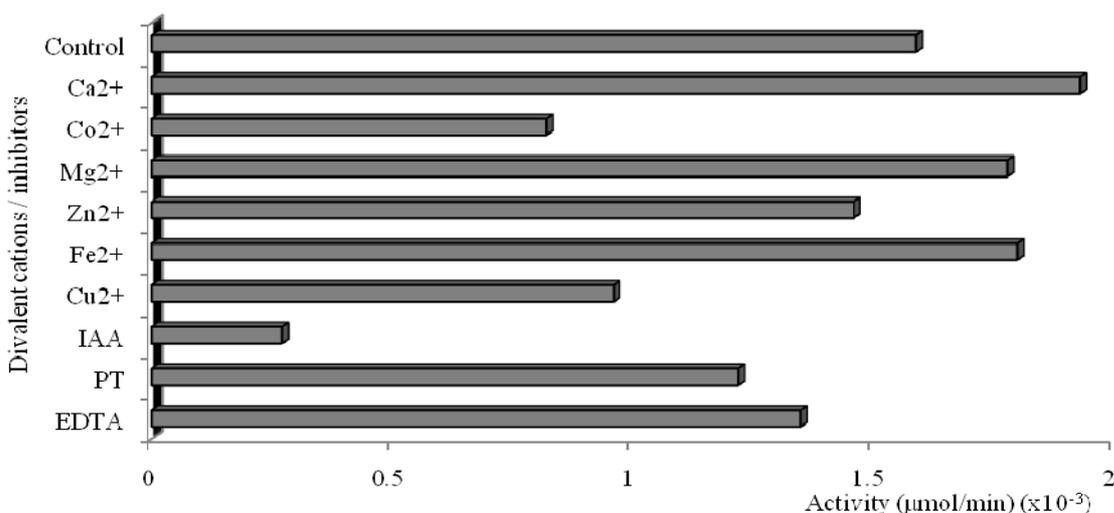


Figure 7. Effects of some divalent cations and inhibitors on the activity of *J. curcas* protease. IAA, PT and EDTA means iodoacetate, 1,10phenanthroline and ethylenediaminetetraacetate respectively. Each point represents the average of three experiments

4. Discussion

Understanding the characteristics of a candidate enzyme for industrial applications is a serious concern for industrial scientists, as it would help to establish the usefulness, or otherwise, of the enzyme in such processes. We report herein the partial characterization of protease from the leaves of *J. curcas*, a plant commonly used as live fence.

The *J. curcas* protease optimal activity at pH 4.0 suggests that the enzyme would maximally hydrolyze proteins in acidic compartments. An acidic pH optimum region contributes to the enhancement of the enzymatic reaction by general acid catalytic mechanism. Optimum activity at acidic pH has been reported for both plant and microbial proteases (O'Donnell et al., 2001; Ire et al., 2011) such as papain and bromelain used in industrial processes. Optimum temperature is an important factor for industrial enzymes selection because most industrial processes occur at slightly higher than physiological temperature. The enzyme had an optimum temperature of

45°C and was stable up to the same temperature in a thermostability studies. This observation is interesting because most industrial enzymes display optimum activity at 40-50°C (Devi, Banu, Gnanaprabhai, Pradeep, & Palaiswamy, 2008; Ire et al., 2011). Moreover, the very low activation energy could imply that proteins degradation by *J. curcas* protease in an industrial process will be thermodynamically favorable because less frequency of collision will be required to surmount the activated complex and form the products. Both observations from the pH and temperature-activity relationships connote that the enzyme has the capacity to withstand the rigors of industrial applications such as food and brewing industries where these variables slightly differ with the physiological environment. On the other hand, it is possible that the *J. curcas* protease display these pH and temperature optima as an adaptational strategy to the extreme climatic and soil conditions that characterize *J. curcas* growth (Devappa, Makkar, & Becker, 2011).

Proteases play a central role in the hydrolysis and mobilization of proteins with different three dimensional structures and therefore other natural proteins were tested as substrates for the *J. curcas* protease. It is noteworthy that all the proteins tested were hydrolyzed by the enzyme although with varying specificities. The observed K_M and V_{max} values are clear indications on the high kinetic efficiency of the enzyme. Previous studies on microbial proteases from *Beauveria* sp (Shankar, Rao, & Laxman, 2011) and *A. niger* (Devi et al., 2008) reported a K_M value of 5.1 mg/ml and 0.8 mg/ml respectively using casein as substrate. Cysteine protease from wheat *Triticumaestivum* had a K_M of 2.8 mg/ml using azocasein as substrate (Fahmy et al., 2004) whereas wheat germ cysteine protease had a K_M of 0.56 mg/ml using casein as substrate (Yang et al., 2011). It seems that the K_M value of the *J. curcas* protease compares relatively less to other known microbial and plant proteases implying higher affinity of this enzyme for the substrates. Furthermore, the K_{cat} value of 0.029 min^{-1} indicates that about 1.78 casein molecules will be hydrolyzed to products per hour at saturation of the enzyme. This could further imply that the enzyme will be useful in industrial processes where the proteins degradation is required at moderate pace. The study of ionizable groups in the active site of *J. curcas* protease demonstrates the presence of groups with pK_{a1} and pK_{a2} of 5.0 and 5.3 respectively which suggests the presence of aspartic acid in and around the active site. The computed enthalpy of ionization is also within the range of beta carboxyl group of aspartic acid (Marangoni, 2003).

Divalent metal ions are involved in enzyme catalysis in a variety of ways which include activation of electrophiles or nucleophiles and bridging an enzyme with substrate together by means of coordinate bonds as well as holding reacting groups in the required three dimensional orientation (Advani, Mishra, Dubey, & Thakur, 2010). Ca^{2+} , Mg^{2+} and Fe^{2+} increased the enzyme activity and considering the relative abundance of these cations in industrial processes, it suggests that the enzyme can easily be activated during the course of an industrial process. The observed effects of other divalent cations (analyzed herein), may suggest a probable modification of the enzyme protein conformation after binding in a different pattern (Ibrahim, Sallau, Salihu, & Onwube, 2011) and hence causing a decrease in enzyme activity. This could further indicate that these divalent metal ions should be avoided during the exploitation of *J. curcas* protease in industrial activities. The protease lost a significant level of activity in the presence of IAA but slightly inhibited by EDTA and PT which suggests that a cysteine residue is involved in the catalytic mechanism. These observations could imply that the enzyme is a thiol (cysteine) protease but does not completely exclude other forms of proteases. Papain and bromelain used extensively in food industries are indeed cysteine proteases (Dubey, Pande, Singh, & Jagannadham, 2007). Thus, the *J. curcas* protease could be of great potential in brewing industries and meat tenderization for the removal of protein hazes.

Some of the prerequisites for the choice of industrial enzymes were investigated in this study and the *J. curcas* protease demonstrated some properties closely similar to other known industrial proteases and with exceptionally low activation energy.

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References

- Advani, S., Mishra, P., Dubey, S., & Thakur, S. (2010). Categorical prediction of metal ion mechanisms in the active sites of 17 select type II restriction endonucleases. *Biochemistry and Biophysics Research Communication*, 402, 177-179. <http://dx.doi.org/10.1016/j.bbrc.2010.09.113>
- Capiralla, H., Hiroi, T., Hirokawa, T., & Maeda, S. (2002). Purification and characterization of hydrophobic amino acid-specific endopeptidase from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates. *Process Biochemistry*, 38, 571-579.

[http://dx.doi.org/10.1016/S0032-9592\(02\)00180-2](http://dx.doi.org/10.1016/S0032-9592(02)00180-2)

- Cheng, K., Lu, F. P., Li, M., Liu, L. L., & Liang, X. M. (2010). Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations. *African Journal of Biotechnology*, *9*, 4942-4953.
- Devappa, R. K., Makkar, H. P. S., & Becker, K. (2011). Jatropha Diterpenes: a Review. *Journal of American Oil Chemical Society*, *88*, 301-22.
- Devi, M. K., Banu, A. R., Gnanaprabhal, G. R., Pradeep, B. V., & Palaniswamy, M. (2008). Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus niger* and its compatibility with commercial detergents. *Indian Journal of Science and Technology*, *1*, 1-6.
- Dubey, V. K., Pande, M., Singh, B. K., & Jagannadham, M. V. (2007). Papain-like proteases: Applications of their inhibitors. *African Journal of Biotechnology*, *6*, 1077-1086.
- Fahmy, A. S., Ali, A. A., & Mohamed, S. A. (2004). Characterization of a cysteine protease from wheat *Triticum aestivum* (cv. Giza 164). *Bioresource Technology*, *91*, 297-304.
- Gubitz, G. M., Lischnig, T., Stebbing, D., & Saddler, J. N. (1997). Enzymatic removal of hemicelluloses from dissolving pulps. *Biotechnology Letters*, *19*, 491-495.
- Gupta, R., Beg, Q. K., & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, *59*, 15-32.
- Ibrahim, M. A., Sallau, A. B., Salihu, A., & Onwube, K. C. (2011). Partial characterization of phospholipase A₂ from the erythrocytic stage of *Plasmodium berghei*. *Asian Journal of Biochemistry*, *6*, 208-213.
- Ire, F. S., Okolo, B. M., & Moneke, A. A. (2011). Purification and characterization of an acid protease from *Aspergillus carbonarius*. *African Journal of Food Sciences*, *5*, 695-709.
- Kaneda, M., Yonezawa, H., & Uchikoba, T. (1997). Purification and some properties of protease from the sarcocarp of musk melon. *Biosciences, Biotechnology and Biochemistry*, *61*, 2100-2102.
- Li, C., Cao, X., Gu, Z., & Wen, H. (2011). A preliminary study of the protease activities in germinating brown rice (*Oryza sativa* L.). *Journal of the Science of Food and Agriculture*, *91*, 915-920.
- Marangoni, A. G. (2003). *Enzyme kinetics, pH dependence of enzyme catalysed reactions*. John Wiley, New Jersey, pp. 79-84.
- O'Donnell, D., Wang, L., Xu, J., Ridgway, D., Gu, T., & Moo-Young, M. (2001). Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. *Biochemical Engineering Journal*, *8*, 187-193.
- Shankar, S., Rao, M., & Laxman, R. S. (2011). Purification and characterization of an alkaline protease by a new strain of *Beauveria* sp. *Process Biochemistry*, *46*, 579-585. <http://dx.doi.org/10.1016/j.procbio.2010.10.013>
- Soares, V. F., Castilho, L. R., Bon, E. P., & Freire, D. M. (2005). High-yield *Bacillus subtilis* protease production by solid-state fermentation. *Applied Biochemistry and Biotechnology*, *121*, 311-319.
- Subramani, T., Chandrashekharaiah, K. S., Swamy, N. R., & Murthy, S. K. R. (2012). Purification and characterization of carboxylesterase from the seeds of *Jatropha curcas*. *Protein Journal*, *31*, 120-128.
- Tang, X. M., Lakay, F. M., Shen, W., Shao, W., Fang, H., Prior, B. A., Wang, Z., & Zhuge, J. (2004). Purification and characterization of an alkaline protease used in tannery industry from *Bacillus licheniformis*. *Biotechnology letters*, *26*, 1421-1424.
- Tunga, R., Shrivastava, B., & Banerjee, R. (2003). Purification and characterization of a protease from solid state cultures of *Aspergillus parasiticus*. *Process Biochemistry*, *38*, 1553-1558. [http://dx.doi.org/10.1016/S0032-9592\(03\)00048-7](http://dx.doi.org/10.1016/S0032-9592(03)00048-7)
- Yang, R., Song, J., Gu, Z., & Li, C. (2011). Partial purification and characterization of cysteine protease in wheat germ. *Journal of the Science of Food and Agriculture*, *91*, 2437-2442.