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Production and Characterization of Amino Peptidase from Marine *Aspergillus flavus*

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Abstract: Amino peptidases have many important commercial applications. The food industry uses them for debittesing and improving the functional properties of protein products and the flavor development in cheese. In pharmaceutical industry inhibitors developing against this enzyme find application as drug. Amino peptidase is also used for peptide sequencing and in processing of recombinant proteins. The different species of fungi were isolated from marine soil sample and the *Aspergillus flavus* was dominantly present. The amino peptidase activity measured at different pH and temperatures was found to be maximal at pH 7 and temperature 45°C. The maximum amino peptidase activity was found to be produced with the use of maltose and potassium nitrite. The molecular weight of the enzyme was determined by SDS-PAGE was found to be 120 Kilodaldon.

Key words: Amino Peptidases · *Aspergillus flavus* · SDS-PAGE · PH · Temperature · Carbon · Nitrogen

INTRODUCTION

Enzymes are proteins that catalyze chemical reactions with the basic function of an enzyme is to increase the rate of a reaction most cellular reactions occur about a million times faster that they would in the absence of an enzyme. The enzymes are regulated from a state of low activity to high activity and vice versa. The activity of an enzyme depends, at the minimum on a specific protein chain.

All animals, green plants, fungi and bacteria produce enzyme. There are about 3000 known enzymes. They are responsible for all the functions of every organ system in our bodies. Enzymes have molecular weights from about 10000 to over l million daldons. Enzymes can be isolated using various protein purification methods. The purity of an enzyme preparation is measured by determining its specific activity [1].

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. Enzymes are highly specific in their action. On substrate and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cells. All enzymes, which have been purified, are one protein in nature and may or may not possess a non- protect prosthetic group [2].

Aspergillus flavus is a haploid filamentous fungus. *Aspergillus flavus* mainly a sprophyte which obtains its nutrient from the decaying material, but it can also be a pathogen to plant and animals including human. *Aspergillus flavus* belongs to the phylum ascomycota. Ascomycota can reproduce sexually by forming ascospores [3].

Aspergillus flavus as a mold like other molds also grows producing hyphase. The network of hyphae or mycelia is responsible for secreting catabolic enzymes. The enzyme secreted is used to down complex food services. The complex food sources are broken down into small molecules which then absorbed by the mycelium to produce asexual spores called conidia [4]. Conidia are globes, smooth and small with size of 2-2.5 mm in diameter. Proyl amino peptidases (PAP) are widespread in nature but little is known about their biological function and the unique structure of proline prevents its cleavage by many broad-specificity amino peptidases [5]. Many bacteria and fungi that produce PAP are

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pathogenic. A role for PAP in inactivity has been reported [6] and it has also been prospered as a viable drug target [7].

The product of PAP has multiple physiological functions in micro-organisms, plants and animals and its accumulation has been implicated in stress responses in yeast and in the suppression of apoptosis in fungi [8]. PAP is an important enzyme industrially, as fungi that possess intracellular proline amino peptidase-type activity have been extensively used in flavor development of food products [9, 10]. Many food products contain flavors obtained by the hydrolysis of proteins. These peptides and amino acids can taste sweet, sour, or bitter. Mixtures of endoproteases are used in conjunction with exoproteases to improve these food flavors. Exopeptidase can reduce the amount of peptides with undesirable tastes through the removal of single hydrophobic amino acid. Amino peptidases catalyze the cleavage of amino acid residues from the N-Terminus of peptides and protein. Aminopeptidases comprise a large group of enzymes that exist in animal and plant tissues as well as in micro organisms. Leucine amino peptidase metabolizes L- Laucylglycine and other peptides with N-terminal leucine. It also hydrolyzes a large variety of substrates, which do not contain leucyl residues [11].

LAPS are exopeptidases belonging to a class of zinc –requiring metalloproteases. However, a few enzymes fermentation require a cobalt ion for full enzyme activity. LAPS have been isolated from a variety of tissues and organs and it exhibits a variety of physico- chemical properties depending on the enzyme source [12, 13]. Amino peptidase is an exopeptidase that catalyzes the hydrolysis of amino acids residues the aminoterminus of polypeptide chains. Aminopeptidase are widely distributed, ubiquitous in nature and are of critical biological importance because of their role in protein degradation.

The objective of the present study was isolation and identification of fungi from marine soil to select the most efficient one as potent source of amino peptidase enzyme.

MATERIALS AND METHODS

Sample Collection and Preparation: The Marine soil sample was collected from coastal region at Atharampattinam, Thanjavur (Dt). The sample was kept in sterile ploythene bags in refrigerator. One gram of soil sample was mixed with 9 ml sterile distilled water and serial dilution of the sample was made.

Isolation and Identification of Fungi: The technique of James and Natalie [14] was adopted for identification of the unknown isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the mycelium from the fungal cultures was removed and placed in a drop of lactophenol. The mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with x10 and x40 objective lenses respectively. The species encountered were identified in accordance with Cheesbrough [15].

Selection of the Fungus: *Aspergillus flavus* were dominantly presented in marine soil. So it was selected for further studies.

Optimization o f pH for Aminopeptidase Activity: The activity of aminopeptidase was evaluated at different pH values, such as 5, 6, 7, 8 and 9 under assay condition. The Fermentation media with each pH were prepared and inoculated with 0.2 ml of 2% fungus spore suspension. The flasks were incubated at room temperature for 5 to 7 days [16].

Optimization of Temperature for Aminopeptidase Activity: To determine the effect of temperature on aminopeptidase activity, the reaction was carried out at different temperatures such as 25, 35, 45, 55 and 65°C. The fermentation media were inoculated with 0.2 ml of 2% fungus spore suspension. The flasks were incubated at selected temperatures for 5 to7 days.

Optimization of Carbon Source for Aminopeptidase Activity: The activity of aminopeptidase was evaluated with the use of different carbon source, such as glucose, lactose, maltose, sucrose and arabinose. The fermentation media were prepared and carbon sources were added. After the sterilization, the prepared media were inoculated with 0.2 ml of 2% fungus spore suspension. The flasks were incubated at room temperature for 5 to 7 days.

Optimization of Nitrogen Source for Aminopeptidase Activity: The reaction was carried out at different nitrogen source, such as ammonium chloride, ammonium nitrate, sodium nitrate, potassium nitrate and calcium nitrate. The inoculated flasks were incubated at room temperature for 5 to 7 days.

Estimation of Protein: Protein concentrations were determined according of Lowery method [17].

Dialysis: The dialysis bag containing the enzyme solution was tightly tied with bands on both sides and kept immersed in buffer solution (Tris Hcl buffer pH-7) for 5 hours. As the solution enzyme contained a lot of residual ammonium sulphate, which was bound to the enzyme. One way to remove this excess salt was to dialyze the enzyme against a buffer in low concentration.

SDS-PAGE: The molecular weight of the dialyzed aminopeptidase was determined by SDS-PAGE.

RESULTS

Isolation of Fungi from Marine Soil: In direct plating eight species of fungi were isolated from the marine soil. The isolated fungi were *Aspergillus sparsusi, Pencillum janthinellum, Aspergillus ochraceous, Aspergillus flavus, Aspergillus terreus, Asperrgillus koningnii*, *Aspergillus humicola* and *Aspergillus niger*.

Screening of the Fungal Isolates for Amino Peptidase Activity: Amino peptidase production and activity was measured by culturing the fungi. *Aspergillus flavus* was selected.

Optimization of pH for Amino Peptidase Activity: Amino peptidase showed maximal activity when assayed at pH 7 (Table 1).

Optimization of Temperature for Amino Peptidase Activity: The maximal activity of amino peptidase was peaked at 45°C (Table 2).

Optimization of Carbon Source for Amino Peptidase Activity: Maximal activity of amino peptidase was peaked with the use of maltose (Table 3).

Optimization of Nitrogen Source for Amino Peptidase: The maximal activity of amino peptidase was found the highest with use of potassium nitrate (Table 4).

Estimation of Protein at Different pH of Amino Peptidase Production: The highest amount of protein produced by *Aspergillus flavus* was at pH 7 compared to other pH values (Table 5).

Table 1: Optimization of amino peptidase production by *Aspergillus flavus* at different pH

S. No.	pH	Enzyme activity (1U/ml) Mean /Standard deviation
1.	5	0.1245±0.0206
2.	6	0.1710±0.0415
3.	7	0. 3431±0.0529
4	8	0.2246±0.0251
5	9	0.2981±0.3454

Table 2: Optimization of amino peptidase production by *Aspergillus flavus* at different temperatures

S. No	Temperature °C	Enzyme activity (1U/ml) Mean /Standard deviation
1.	25	3.3750±0.1902
2.	35	3.6631±0.4894
3.	45	0.4426±0.1804
4	55	3. 5026±0.1223
5	65	0.2213±0.0829

Table 3: Optimization of different carbon sources for amino peptidase activity of *Aspergillus flavus*

S. No.	Carbon sources	Enzyme activity (1U/ml) Mean /Standard deviation
1.	Glucose	0.0821±0.0086
2.	Maltose	3.5336±0.4905
3.	Lactose	0.3486±0.4384
4.	Sucrose	0.1128±0.0174
5.	Arabinose	0.4426±0.4423

Table 4: Optimization of different nitrogen sources for amino peptidase activity of *Aspergillus flavus*

S. No.	Nitrogen source	Enzyme activity (1U/ml) Mean /Standard deviation
1.	Ammonium nitrate	0.3373±0.0285
2.	Ammonium sulphate	0.2536±0.0568
3.	Sodium nitrate	0.3820±0.5040
4	Potassium nitrate	0.3863±0.0302
5	Calcium nitrate	0.2703±0.0212

Table 5: Estimation of protein at different pH of amino peptidase production

S. No.	pH	Estimation of protein (1U/ml) Mean /Standard deviation
1.	5	0.0356±0.0488
2.	6	0.0123±0.0005
3.	7	0.1533±0.1007
4.	8	0.1201±0.0100
5.	9	0.0116±0.0015

Table 6: Estimation of protein at different temperatures of amino peptidase production.

S. No	Temperature (°C)	Protein(1U/ ml) Mean /Standard deviation
1.	25	0.1841±0.0539
2.	35	0.2463±0.0217
3.	45	0.3538±0.0629
4.	55	0.2525±0.0347
5.	65	0.2586±0.0121

Table 7: Estimation of protein with different carbon sources for amino peptidase production

S. No>	Carbon source	Enzyme activity (1U/ml) Mean /Standard deviation
1.	Glucose	0.0273±0.0058
2.	Maltose	0.1103±0.0110
3.	Lactose	0.0186±0.0066
4.	Sucrose	0.1006±0.0005
5.	Arabinose	0.0353±0.0110

Table 8: Estimation of protein with different nitrogen sources for amino peptidase production

S. No.	Nitrogen source	Enzyme activity (1U/ml) Mean /Standard deviation
1.	Ammonium nitrate	0.1667±0.0540
2.	Ammonium sulphate	0.2081±0.1065
3.	Sodium nitrate	0.1733±0.0551
4.	Potassium nitrate	0.2036±0.0551
5.	Calcium Nitrate	0.1923±0.0855

Table 9: Purification of amino peptidase from *Aspergillus flavus*

Purification level	Volume (ml)	Activity (U/ml)	Specific activity (U/mg)	Protein U/mg	Yield (%)	Purification fold
Crude enzyme extract	100	6.5	3.80	70.3	100	1
Ammonium sulphate fractionation	50	4.30	2.18	55	55	1.2
Dialysis	25	2.82	1.00	15.8	15.8	2.7

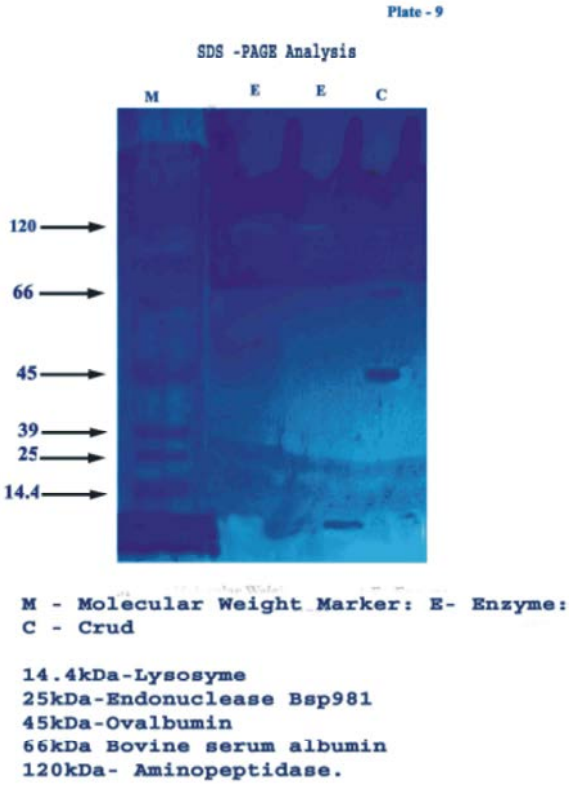

Fig. 1: SDS-PAGE analysis

Estimation of Protein at Different Temperatures of Amino Peptidase Production: The highest amount of protein produced by *Aspergillus flavus* was at 45°C compared to other temperature values (Table 6).

Estimation of Protein with Different Carbon Source for Amino Peptidase Production: The highest amount of protein produced by *Aspergillus flavus* was with the use of maltose compared to other carbon source (Table 7).

Estimation of Protein with Different Nitrogen Source for Amino Peptidase Production: The highest amount of protein produced by *Aspergillus flavus* was with potassium nitrate compared to other nitrogen sources (Table 8).

Purification of Amino Peptidase: The crude enzyme solution prepared from 85% ammonium sulphate saturation of *Aspergillus flavus* was dialyzed against 10mM Tris – Hcl buffer, pH- 7 for 24 hours and it was applied to gel filtration at 4°C, which was previously equilibrated with the same buffer (Table 9).

SDS-PAGE: The amino peptidase extracted from *Aspergillus flavus* had the molecular weight of 981basepir Figure 1.

DISCUSSION

In the present investigation, isolation, production, optimization and characterization of amino peptidase from marine *Aspergillus flavus* was carried out. Eight species of fungi were isolated from marine soil; *Aspergillus flavus, A. ochareceous, Aspergillus sparsuii, Pencillum janthinellum, Aspergillus terreus, Aspergillus Koeningii, Aspergillus humicola* and *Aspergillus niger.* Screening of fungal isolates for amino peptidase production revealed that *Aspergillus flavus* produced highest extracellular amino peptidase activites. *Aspergillus* culture has been used for nonspecific amino peptidase production [18]. On the other hand, Nampoothri and his colleagues have previously screend 28 *Aspergillus* starins and have identified two *A. sojae* strains and one *A. oryaze* strain as being superior LAP producers [19]. Lin *et al*. [20] described the complement of extracellular LAP produced from a submerged fermentation of *A.oryzae* LLI using

chicken meat commercial enzymes LAP, LL1's. LAP specific activity was 0.054Umg-1. The enzyme substrate hydrolysis characteristics were comparable to flavourzyme, a typical fungal complex of exopeptidase and endopeptidase used in industrial food processing.

In the present study, the *Aspergillus flavus* was tested for production of aminopeptidase. Aminopetidase production and activity was measured by culturing the fungi.

In the present study, the production of amino peptidase by *Aspergillus flavus* determined by assay the enzyme at different temperatures (25°C, 35°C, 45°C, 55°C, 65°C,) was peaked at 45°C. *A. oryzae* LL1 and LL2 cultures yielded maximal LAP production at 30°C. At 25, 35 and 40°C extracellular LAP activity in *A. oryzae* LL1 cultures was decreased to 58.5, 8.5 and 1.4% of the maximal activity respectively [21].

Elinbanu *et al*. [22] reported that the effect of initial pH of the medium on LAP yield was examined by using media of pH 4.0 -7.0. LAP activity was observed for mycelial biomass and LAP production of *A.oryzae* LLI between 4.5 and 5.5. Although, the optimum initial pH for LAP production of *A.oryzae* LL2 was between 5.5 and 7.0 however the LAP yield tended to decline significantly when pH was below.

In our study amino peptidase showed maximal activity, when assayed at a pH-7. The activity of aminopeptidase was estimated at different intervals of growth and the activity of enzyme gradually increased with increasing pH till pH 7, then enzyme activity decreased with increase of pH.

Both carbon and nitrogen sources are important for the growth of the strains and for LAP production. This characteristic is reflected in the high levels of hydrolytic enzyme activities that have been found in the mycelia lysates and culture filtrates of many fungi. Previous work has shown that peptide hydrohysates such as casitone (as enzymic hydrolysate of casein) are excellent inducers of a major extracellular peptidase *T. emersonii* [23].

In this investigation, the molecular weight of the amino peptidase from *Aspergillus flavus* was found to be albumin. The molecular weight of the aminopeptidase from *Aspergillus flavus* was found to be 120 Kilodaldon.

CONCLUSION

The highest Protein activity in aminopeptidase enzyme from various parameters. The molecular weight of enzyme was determined by SDS-PAGE. The standard protein marker ranging from catalase (160 KDa). The molecular weight of the aminopeptidase from *Aspergillus flavus* was found to be 120 kD.

REFERENCES

1. Grisham, G., M. Charles and H. Reginald, 1999. Biochemistry Rhiladophia. Sounders college Publication, 7: 18-20.
2. Under Kolfer, L.A., R.R. Barton and S.S. Rennert, 1957. Production of microbial enzymes and their application. Microbiological Process Report, 6: 212-221.
3. Amaike, Saori and Nancy P. Keller 2011. Aspergillus flavus. The Annual Review of Phytopathology, 49: 107-133.
4. Wilson, D.M., R.C. Gueldner, J.K. Mckinney, R.H. Lievsay, B.D. Evans and R.A. Hill, 1981. Effect of *â*-lonone on *Aspergillum flavus* and *Aspergillus parasiticus* growth, sporulation, morphology and aflatoxin production. Journal of the American Oil Chemists Society, 58: A959-A961.
5. Cunnigham, D.F. and B. Connor, 1997. Proline specific peptidases. Brioche. Biopsies. Act, 1343: 160-186.
6. Zhang, L., L. Jiao Wang and R. Fang, 2007. A proline aminopeptidase gene unregulated in plantar by a LUXR homologue is essential for pathogen city of xanthomonas campestris, pU. Campestris. Mol. Microbial., 65: 121-136.
7. Felipe, M.S., R.U. Andrade, F.B. Arraes, A.M. Nicola, A.Q. Maranhao, F.A. Torres, I. Sliva Pereira, M.J.P. Fonseca, E.G. campes and other authors 2005. Transcriptional profiles of the human pathogenic fungus paracoccidioides brasiliensis in mycelium and yeast cells. J. Biol. Chem., 280: 24706-24714.
8. Takagi, H., 2008. Proline as a stress protecant in yeast, physiological functions, Metabolic regulation and biotechnological applications. APPl. Microbial. Biotechnol., 81: 211-223.
9. Bolumar, T., Y. Sanz, M.C. Aristoy and F. Toldra, 2003. Purification and characterization of a polyl aminopeptidase from Debaryomyces hansenii. Appl. Environ. Microbiol., 69: 227-232.
10. Fuke, Y. and H. Matsuoko, 1993. The purification of prolyl aminopeptidase from pencillium camemberti. J. Dairy. Sci., 76: 2478-2484.
11. Smith, E.L. and R.L. Hill, 1960. In the enzyme. (Boyer, P.D., H. Lardy and K. Myrvack., Eds). Acad. Press. New York, 2(4): 37-62.

12. Taylor, A., 1993. Aminopeptitase to words a mechanism of action. Trends. Brioche. Sci., 12: 167-172.
13. Van Wart, H.E. and Lin, 1981. Metal binding stoichiometry and mechanism of metal iron modulation of activity of porcine kidney leucine aminopeptidase. Biochemistry, 20: 5682-5689.
14. James, G.C. and S. Natalie, 2001. Microbiology. A laboratory Manual (Ed.), pp: 211-223.
15. Cheesbrough, M., 2000. District Laboratory Practice in Tropical Countries Part 2, Cambridge University Press, Cambridge, pp: 47-54.
16. Junichiro Marui, Mayumi M. Morita, Sawaki Tada, Ryota Hattori, Satoshi Suzuki, Hitoshi Amano and Hiroki Ishida, 2012. Enzymatic properties of the glycine d-alanine aminopeptidase of *Aspergillus oryzae* and its activity profiles in liquid-cultured mycelia and solid-state rice culture (rice koji). Applied Microbiology and Biotechnology, 93: 655-669.
17. Lowry, O.H., N.S. Rosebreugh, A.L. Ferr and R.J. Randal, 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193: 265-275.
18. Blinkousky, A.M., T. Byun, K.M. Brown, E.J. Golightly and A.V. Klotz, 2000. A non-Specific aminopeptidase from Aspergillus. Brioche. Biophys. Acta., 1480: 171-181.
19. Nampoothiri, K.M., U. Nagy, K. Kovacs, G. Szakacs and A. pandey, 2005. L-leucine aminopeptidase production by filamentous Aspergillus fungi. Applied Microbiology, 41: 498-504.
20. Lin, S.J., Y.H. Chen, L.L. Chen, H.H. Feng, C.C. Chen and W.S. Chu, 2008. Large scale production and application leucine aminopeptidase produced by Aspergillus oryzae. LLI for hydrolysis of chicken breast meat. Eur. Food. Res. Techno., 227: 159-165.
21. Shie-Jea. Lin-Yi-Hong chen and Li-Lin chen, 2008. Large scale production and application of leucine aminopeptidase produced by Aspergillus oryzae LLI for hydrolysis of chicken breast meast. Eur. Food. Res. Technol., 227: 159-165.
22. Elinbanum, S., H. Ferreyra, G. Ellenrieder and C. Cuevas, 2002. Production of Aspergillus terreus alpha-1-rhamnosidase by solid state fermentation. Appl. Microbial., 34: 6771.
23. Anthony, J. Donoghue, Cathal S. Mahon, David H. Goetz, James M. Malley, Denise M. Gallagher, in Zhou, Patrick G. Murray, Charles S. Craik and Maria G. Tuohy, 2008. Inhibition of a Secreted Glutamic Peptidase Prevents Growth of the Fungus *Talaromyces emersonii.* The Journal of Biological Chemistry, 283: 29186-29195.