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RESEARCH ARTICLE

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PRODUCTION AND PARTIAL PURIFICATION OF LEUCINE AMINOPEPTIDASE FROM *AEROMONAS PROTEOLYTICA* (ATCC 15338)

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ABSTRACT

Aeromonas proteolytica (ATCC 15338) was grown at flask level and fermenter level in different concentrations of LB. LB at 4 % was found to be optimum for maximum production of Aminopeptidase. At flask level, 30 °C and 180 rpm was found to be optimum. In fermenter level, Aminopeptidase was successfully produced at 30 °C, 700 rpm, pH 7.0 ± 0.05 and airflow of 0.5 LPM. Partial purification of the protein was achieved by ion exchange chromatography on DEAE sepharose. Aminopeptidase was eluted between 170 mM and 230 mM of NaCl concentration in a gradient elution process. Buffer exchange was done by gel filtration chromatography (Sephadex G25). In reverse phase high performance liquid chromatography (RP-HPLC), the retention time of standard and that of the partially purified Aminopeptidase was recorded at ~12 min. Molecular weight and iso electric point of standard (Aminopeptidase from Sigma) and partially purified Aminopeptidase were found to be ~30 kD and 3.5 respectively.

KEYWORDS

Aeromonas Proteolytica, ATCC 15338, Aminopeptidase, media optimization, molecular weight

INTRODUCTION

There has been a great interest in the bacterial proteases, which finds immense applications from industrial point of view and also in the elucidation of enzyme-functioning mechanisms. Various kinds of proteases have been present in micro-organisms. Microbial proteases have been widely used for various fermentation-based applications and production of food and medicaments, because of relatively easy availability of large quantities at economical costs.

Industrially important class of bacterial proteases has always been of great importance. Leucine Aminopeptidases (LAP) are exo-peptidases which remove the N-terminal L-Leucine from peptide substrate[1]. Several Leucine Aminopeptidases are of microbial origin: *Bacillus subtilis*[2], *Aeromonas Caviae*[3], *Aeromonas Proteolytica*[4], Escherichia coli[5], marine Pseudomonas[6],Streptomyces *lividans* [7] particularly from genus S*treptomyces*[8-11]. Several genes encoding for bacterial LAP have also been isolated[12-14]. Aminopeptidases are useful in preparation of protein hydrolysates[3]. This paper provides an insight on production of Aminopeptidase from *Aeromonas proteolytica* (ATCC 15338), partial purification and identification.

MATERIALS AND METHODS

Chemicals used: L-Leucine p-Nitroanilide, Tricine, Bovine Serum Albumin (BSA), Aminopeptidase, Terrific broth, and Tris from Sigma, USA; LB, APS super broth , LB agar and Marine broth (Difco, USA), Sodium di-hydrogen Phosphate, Acetonitrile, glycerol and Sodium Chloride from Merck; Chemical for SDS PAGE from Sigma, USA.

Microorganism used:

Wild strain of *Aeromonas proteolytica* was obtained from American Type Culture Collection (ATCC), number 15338. Culture was grown on Petri plate containing LB Agar at 37 °C; single colony was picked up and suspended in LB media for growth. When optical density of the culture reached ~1.0, sterile 80 % glycerol was added in a way that the final concentration of glycerol was 20 %. Culture was stored in Cryo vials at -80 °C for further use.

Quantification using enzymatic method:

Enzymatic assay for Aminopeptidase quantification was carried out using a protocol developed by Sigma (EC 3.4.11.10). For the assay, Aminopeptidase from Sigma was used (Cat. No. A8200). The only difference in this experiment is that the range used is of 0.001 to 0.005 units of Aminopeptidase whereas in the Sigma protocol, it was 0.002 to 0.004 units. Aminopeptidase activity was measured spectrophotometrically at 405 nm using a synthetic substrate, L- Leucine p-Nitroanilide. In the presence of aminopeptidase it is cleaved in to L-Leucine and p-Nitroaniline.

Final assay concentrations:

In a 1.0-mL reaction mix, the final assay concentrations are 20 mM Tricine, 0.4 % (v/v) methanol, 0.18 mM L- Leucine p-Nitroanilide, 0.005 % (w/v) bovine serum albumin, and 0.001 - 0.005 unit Aminopeptidase.

Unit definition: One unit will hydrolyze 1.0 µmoles of p-nitroanilide to L-Leucine and p-nitroaniline per minute at pH 8.0 at 25°C.

CALCULATION

$$\text{Units/ml enzyme} = \frac{(\Delta A_{405nm}/\text{min Test} - \Delta A_{405nm}/\text{min Blank})\ (1)\ (df)}{(10.8)\ (0.1)}$$

1 = Total volume (in milliliter) of assay
df = Dilution factor
10.8 = Millimolar extinction coefficient[15] of p-Nitroaniline at ΔA_{405nm}
0.1 = Volume (in milliliter) of enzyme used

Process optimization at flask level:

Growth kinetics study at different temperatures in marine broth:

For growth kinetics study, two conical flasks (500 mL), each containing 200 mL media, were used and were prepared by dissolving 7.5 g Marine Broth (MB, Difco) along with 2.0 g NaCl in water for injection (WFI). pH of the media in the flasks was adjusted to 7.6 ± 0.2 and autoclaved at 121°C for 15 min. After sterilization when the temperature of media reached to room temperature, 200 µL of culture (from frozen Vial) of *Aeromonas proteolytica* was added to both the flasks under Biosafety hood and incubated in two different incubator shakers (New Brunswick scientific) at 180 rpm. Temperature of incubators was maintained at 30 °C and 37 °C respectively. Optical density was measured at 1 hour interval up to 14 hours (till stationary phase arrived).

Pre-inoculum (seed) preparation:

Pre-inoculum was prepared in Marine broth. Marine broth (200 mL; 37 g/L) along with 1 % NaCl was prepared in a conical flask and autoclaved at 121°C for 15 min. To the autoclaved media (when temperature reached to room temperature) 200 µL of culture (from frozen Vial) was inoculated and the flask was incubated in an incubator shaker at 180 rpm and 30 °C till its OD reached to approximately 1.0 ± 0.1.

Fermentation media selection at flask level:

Different types of fermentation media (200 mL) were prepared (Table 1) in 500-mL conical flasks and were autoclaved. When the pre-inoculum OD reached to 1.0 ± 0.1, required quantity of pre-inoculum was transferred to fermentation media in a way that the initial OD in the media was 0.01. Flasks were incubated in the incubator shaker at 180 rpm and 30°C. OD was checked at different intervals till the constant OD was recorded (17h).

Table 1
Details of media composition in flask level study for media screening

Parameter/ Media	LB	Marine broth	Terrific Broth	APS Super broth
Media concentration* (g/L)	20	37	47.8	49.1
Media volume	200 mL			
Media pH	7.0 ± 0.1			
rpm	180			
Temperature	30 °C			
Total incubation time (h)	17			

** These media concentrations were selected based on manufacturer's recommendation.*

Optimization of concentration of LB:
Different concentrations of LB (Table 2) were evaluated for the growth of *Aeromonas proteolytica* and production of the enzyme. Pre-inoculum used for this study was also similar to that used in the media selection study in flask level. Enzyme production and growth kinetics were observed and recorded in this study.

Table 2
Details of media composition in flask level study during LB concentration optimization

Parameter/ Media	LB			
	2 %	4 %	6 %	8 %
Media concentration (g/L)	20	40	60	80
Media volume	200 mL			
Media pH	7.0 ± 0.1			
rpm	180			
Temperature	30 °C			
Total Incubation time (h)	17			

Fermentation Process at fermenter level:
As 4 % LB yielded best result, fermentation process was developed using the same media concentration. Fermenter used for the study was Sartorius-Biostat B plus having 6.6 L capacity. Fermentation media (4 L) containing 4 % LB and 0.6 mL/L antifoam was prepared and autoclaved at 121 °C for 20 min. Fermentation process parameters during the study were 700 rpm, pH 7.0 ± 0.05, temperature 30 °C and airflow 0.5 LPM. When the pre-inoculum OD reached to 1.0 ± 0.1, required quantity of pre-inoculum was added in such a way that the initial OD in the fermentor was 0.01. During the entire process, pH was maintained with autoclaved 2 M NaOH and 9 % HCl. Enzyme activity and OD of cells was measured at regular intervals of 1 hour upto 23 hours.

Isolation of Aminopeptidase:
After 22 hours of fermentation, batch was harvested and broth was centrifuged at 10800 g for 20 min. Supernatant was collected and stored at (2-8) °C.

Purification of Aminopeptidase:

Ultrafiltration:
The output obtained from the fermentation harvest (4 L) was concentrated ten-folds using 10 kDa slice cassette (Sartorius) with cross-sectional area 0.1 m^2, tangential flow filtration system (TFF, Sartorius). The overall flow-rate of ultra filtration system was maintained at 500 mL/min. The permeate pressure was 0 bar, retentate pressure 0.4 bar and the input pressure was set to 0.6 bar.

Diafiltration:
The buffer exchange was done using Diafiltration process. The ten-fold concentrated ultra filtration output (UF-OP) was diluted to ten-fold with 50 mM Tris-HCl buffer (pH 8.0). The remaining process was kept similar to ultrafiltration process. After ten-fold dilution and Diafiltration, if conductivity was more than 3-4 ms/cm, then the entire protein solution was diluted with 50 mM Tris-HCl buffer (pH 8.0) and the process was continued till the conductivity was obtained in the range of 3-4mS/cm.

Ion-Exchange Chromatography:

After Diafiltration step, the output was filtered using 0.45-µm disc filter (PALL Life Sciences). Chromatography was carried out at room temperature using a XK26 column (2.6 x 20 cm) packed with DEAE-Sepharose Fast Flow (Amersham bioscience) with a bed volume of about ~30 cm^3 having bed height 5.5 cm and connected to an ÄKTA prime (chromatographic system). Column equilibration was done with 50 mM Tris-HCl buffer (pH 8.0). Diafiltration output was loaded to the column. The column was washed with 3 CV (column volume) equilibration buffer (50 mM Tris-HCl, pH: 8.0) to remove any unbound proteins. The elution of protein of interest was done with a linear gradient of 0-0.5 M NaCl with 5 CV elution buffer (50 mM Tris-HCl and 1 M NaCl, pH 8.0). Detection was done at 280 nm. Different fractions were collected and checked for enzymatic activity of Aminopeptidase using the colorimetric based enzymatic assay.

Thermal Incubation:

Active fractions (which showed enzymatic activity) from ion-exchange chromatography output were subjected to thermal incubation for 45 minutes at 70 °C. It was then centrifuged at 10800 g for 20 minutes and supernatant was collected.

Gel Filtration Chromatography:

Gel filtration Chromatography was carried out in XK26 column (2.6 x 40 cm) from Amersham biosciences containing sephadex G-25 matrix. Column was packed at a flow rate of 15 mL/min having total bed volume ~160 cm^3 with bed height 30 cm. Column was equilibrated at flow-rate of 10 mL/min with 50 mM Tris-HCl, pH: 8.0. This flow-rate was maintained throughout the GFC process. Active fractions showing enzymatic activity were loaded to the GFC column in a way that loaded volume was not more that (15-20) % of total bed volume. Detection was done at 280 nm. Peak was collected when the main peak reached 25 mAu in upward direction and collection was continued till the absorbance reached to 25 mAu in downward direction.

Identity test for Aminopeptidase RP-HPLC:

Zorbax 300 SB-CN column (4.6 × 150 mm) and HPLC (Agilent 1200 series) were used for the analysis**.** Initially the column was equilibrated with 10 % buffer B (20 mM NaH_2PO_4 in 60 % ACN) and 90 % buffer A (20 mM NaH_2PO_4 in WFI, pH 4.5) for 5 minutes. Then the gradient of elution buffer (buffer B) was increased to 1 % per minute, up to 20 minutes. Flow-rate was maintained at 1 mL/min throughout the method. Absorbance was detected at 215 nm. Main peak (peak 3) was collected, lyophilized and analyzed by SDS PAGE and isoelectric focusing.

SDS-PAGE:

SDS PAGE was carried out as an identity test for the characterization of Aminopeptidase. During RP-HPLC run, different peaks were collected and labeled as Peak 1, Peak 2 and Peak 3 (refer to Figure 7). Samples of each peak were lyophilized using a freeze dryer and then reconstituted with 50 mM Tris-HCl buffer (pH: 8.0) and loaded on to 12 % gel. Electrophoresis was carried out at 150 V till the dye came out of the gel. The gel was silver stained for visualization of the protein bands.

IEF:

IEF was carried out on Phast System (Amersham bioscience) to identify the pI and to compare it with standard Aminopeptidase. Gel was silver stained for visualization of protein bands.

RESULTS AND DISCUSSION

Quantification methods:

Standard curve was prepared to quantify the amount of Aminopeptidase produced during fermentation and to calculate the step recovery. Good linearity (R^2=1) (Figure 1) within 0.001 to 0.005 units of Aminopeptidase was seen.

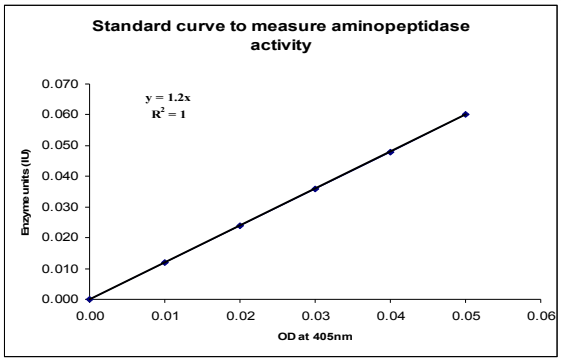

Figure 1
Standard curve for aminopeptidase estimation

Growth kinetic study and production of Aminopeptidase at different temperatures in marine broth media:

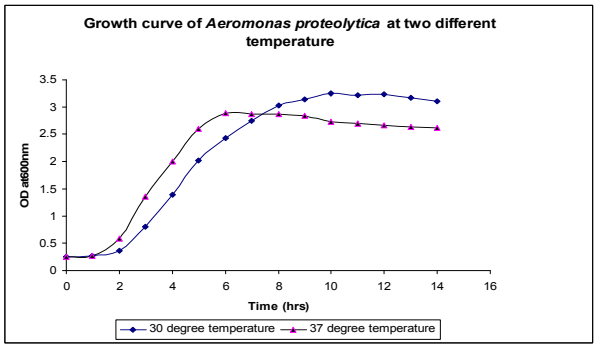

Figure 2
Growth kinetics of Aeromonas proteolytica in Marine broth at different temperatures

From the above curve (Figure 2), it was observed that in marine broth media, as compared to 30 °C (2 hours), the lag phase was short at 37 °C (1 hour). After 5 hours of incubation, cells entered stationary phase at 37 °C whereas at 30 °C, cells took 8 hours to enter the stationary phase. Maximum specific growth, obtained at 37 °C was 0.84 h^{-1} and at 30 °C, it was 0.81 h^{-1}. From the above study, it was found that the maximum (overall) growth occurred at 30 °C.

Aminopeptidase production in different media:
Different media (LB, Marine broth, TB and APS Super broth) used for the optimum production of aminopeptidase shows that LB media is found to be superior to others (Table 3).

Table 3
Comparison of aminopeptidase production and growth obtained in different media

Performance /Media	LB	Marine Broth	TB	Super Broth
Final OD	5.36	3.13	5.68	6.38
Maximum IU/mL produced at different time period	70.0	0	6.0	7.6

Growth kinetic study and Aminopeptidase production at different concentrations of LB media:

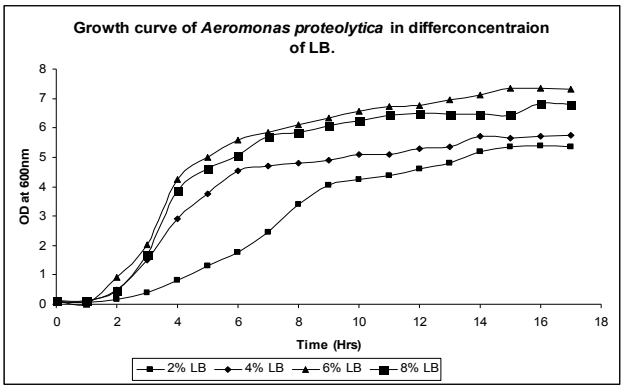

Figure 3

Growth kinetics of Aeromonas proteolytica in different concentrations of LB

From the above graph (Figure 3) it was found that maximum growth was obtained in 4 % and 6 % of LB. As in 4 % LB Aminopeptidase production was maximum, fermentation process was optimized in 4 % LB (Table 4).

Table 4

Maximum OD and aminopeptidase yield obtained during optimization of LB concentration

Performance /Media	2 % LB	4 % LB	6 % LB	8 % LB
Final OD	5.36	5.75	7.33	6.78
Maximum IU/L produced at different time period	70	108	50.4	6

Fermentation:

During the fermentation process, it was found that production of Aminopeptidase started after 10 hours (Figure 4). Production of enzyme increased with increase in time till 22 hours. After 22 hours production of enzyme decreased gradually. Maximum Aminopeptidase production was obtained at 22 hours (258 IU/L). In fermenter maximum OD achieved was 10.8 and biomass production was 30 g/L. From the above experiment, it is concluded that 22 hours of fermentation was optimum for maximum Aminopeptidase production.

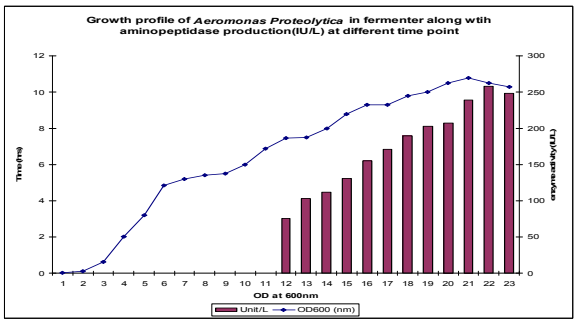

Figure 4

Growth profile of aminopeptidase in fermenter along with aminopeptidase production at different time points

Ultrafiltration and Diafiltration:

During Ultrafiltration and Diafiltration, it was found that only ~6 % of Aminopeptidase was lost in terms of IU (Table 5).

Ion exchange Chromatography:

Different peaks were collected and studied for the presence of Aminopeptidase. From DEAE (ion exchange) chromatography, it was found that Aminopeptidase eluted between 170 mM and 230 mM of NaCl (Figure 5) at the provided conditions. The total recovery at this step was 86.9 %. Fractions showing Aminopeptidase activity was collected and incubated in a hot water bath at 70°C for 45 minutes. It was centrifuged at 10800 g for 20 minutes to remove the impurity. The total recovery at this step was 52.5 %.

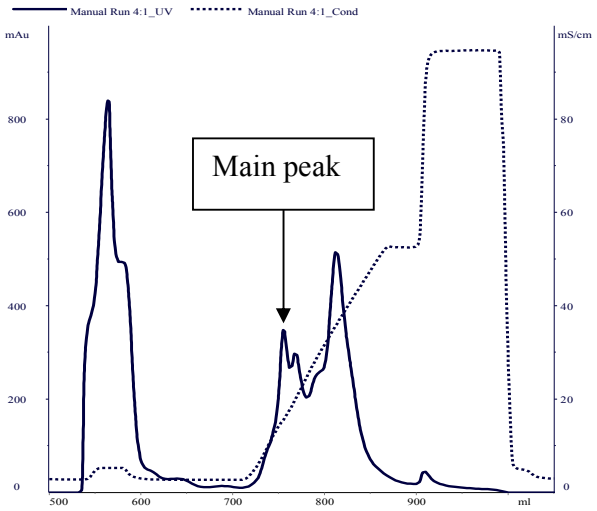

Figure 5
Chromatogram of Ion exchange (DEAE) chromatography

Gel filtration chromatography:

Gel filtration chromatography was carried out for buffer exchange. From Figure 6 it is found that during Gel filtration salts were removed completely and Aminopeptidase was stored in 50 mM Tris-HCl, pH: 8.0. Total recovery at this step was 31.9 %.

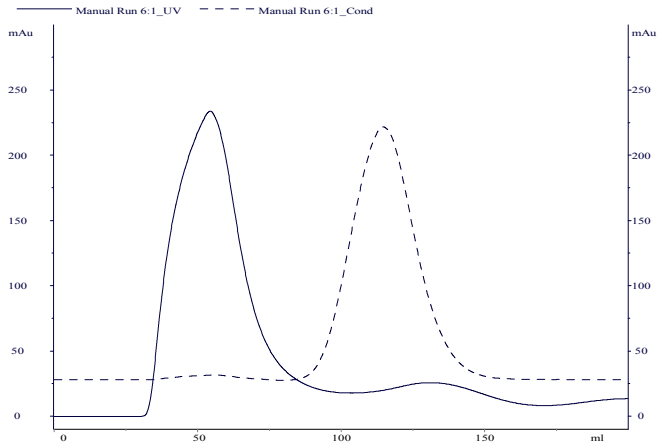

Figure 6
Chromatogram of Gel filtration chromatography

Table 5
Step yield of downstream steps and final recovery

Step	Volume (mL)	Total Unit	Recovery relative to 1st step (%)
Harvest	4000	948	100
UF OP	400	938	98.9
DF OP	400	896	94.5
DEAE OP	240	824	86.9
After Heating	240	498	52.5
GFC	330	302	31.9

Identity test:

From RP-HPLC analysis it was found that the Aminopeptidase was getting eluted at ~17 % of elution buffer (Figures 7 and 8). Retention time of Aminopeptidase was ~12 minutes in the provided conditions. Different peaks (pre peak) were collected, lyophilized and subjected to SDS PAGE under reducing conditions. From the SDS PAGE (Figure 9) it was found that Peak 3 matched with the standard. Peaks 1 and 2 did not contain Aminopeptidase. Through reducing SDS PAGE molecular weight of Aminopeptidase was found to be ~30 kD (between 25 kD and 37 kD in the figure). The same observations are also observed by different authors[3-4].which also matched with Sigma product information sheet.

From the results of isoelectric focusing (Figure 10), isoelectric point of Aminopeptidase was found to be ~3.5 for standard and for the sample (lyophilized peak 3). Product information sheet of aminopeptidase (Sigma) also revealed the same.

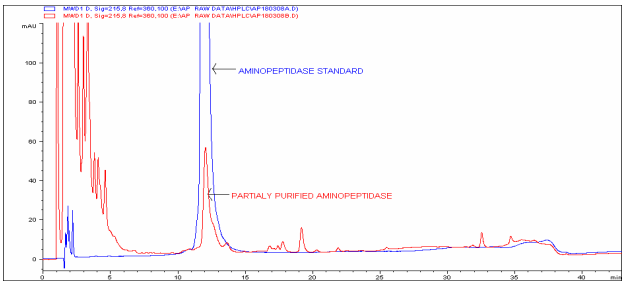

Figure 7
Comparison of retention time of standard Aminopeptidase from Sigma with the purified Aminopeptidase

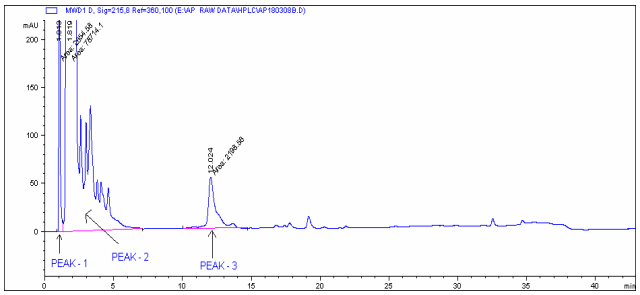

Figure 8
Peaks labeled as 1, 2, and 3 (in RP-HPLC chromatogram) which were collected and lyophilized

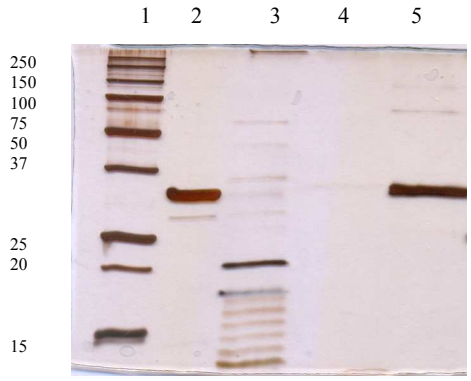

Figure 9

SDS PAGE showing impurity profile of different peaks collected from RP-HPLC. Sample details are, Lane 1 molecular weight marker, Lane 2 STD, Lane 3 lyophilized sample of Peak 1, Lane 4 lyophilized sample of peak 2 and Lane 5 contains lyophilized sample of Peak 3.

SDS PAGE (Figure 9) illustrates that Peak 1 (Lane 3) contains impurities and Peak 2 (Lane 4) can be considered as a buffer peak as it does not contain any protein bands. Peak 3 (Lane 5) contains Aminopeptidase and molecular weight matches with standard Aminopeptidase.

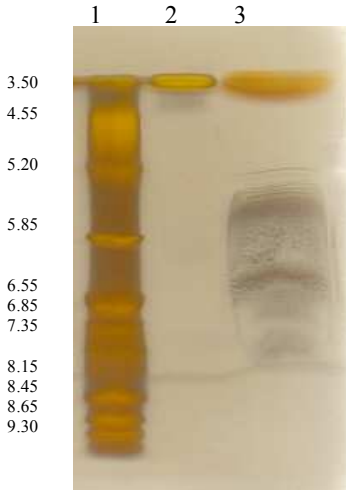

Figure 10

Iso electric focusing of Aminopeptidase. Sample details are as follows: Lane1-IEF Marker, Lane 2-STD and Lane 3-lyophilized sample of peak 3 (RP-HPLC chromatogram).

CONCLUSION

LB media was found to be better than Marine broth, Terrific broth and APS Super broth. Among different concentrations of LB, 4 % was found to be most suitable for maximum Aminopeptidase production. *Aeromonas proteolytica* secretes Aminopeptidase in growth media successfully at 700 rpm, pH 7.0 ± 0.05, temperature 30 °C and airflow 0.5 LPM. Using DEAE as an anion exchanger Aminopeptidase was successfully purified to 70 % with gradient elution by NaCl from 170 mM to 230 mM. Aminopeptidase identity was confirmed with RP-HPLC, SDS PAGE and Isoelectric focusing using standard Aminopeptidase from Sigma.

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