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Valorization of Catla Visceral Waste by Obtaining Industrially Important Enzyme: Trypsin

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Abstract

The fish visceral waste is currently posing a major problem to fisheries and environment sustainability. India has generated fish waste around 50% of the production in the year 2015-16. The indiscriminate practices of waste disposal cause physical and ecological imbalances in the ecosystem. Hence, its management has become very important. The visceral organs are known sources of biologically active compounds therefore, value-added products can be recovered. The present study focused on isolation of visceral protease of popular fish species, Catla catla. The proteins were precipitated using various precipitating agents among which cold acetone was found best (152.93mg ml-1 protein). The kinetic study of the enzyme was performed using BAPNA substrate and enzyme activity (15.54 U ml-1), Km (2.47mM) and Vmax (0.40µm-1 min-1) were calculated. The molecular weight of the protease was 24KDa, optimum pH was 8.5, and optimum temperature was 40ºC. The enzyme was found 19.3% similar with mammalian (bovine) trypsin. Henceforth, the enzyme was thought to solve the fish visceral waste problem with an ecological approach.

Keyword: Fish visceral waste; Protease; Trypsin; Enzyme kinetics; Lineweaver-Burke plot; SDS-Page

Abbreviations: BSE: Bovine Spongiform Encephalopathy; BSA: Bovine Serum Albumin; TIC: Total Ion Current Chromatogram

Introduction

Trypsin (EC 3.4.21.4) a serine peptidase, is prevalent throughout the animal kingdom. It digests the proteins and peptides by attacking at the Lys and Arg residues from their carboxyl end. It is synthesized as trypsinogen, as an inactive form by the pancreas into the intestine where it activates other intestinal enzymes along with itself Kanno [1]. The catalytic triad of trypsin is made up of Ser, His and Asp and has a negative charged pocket to recognize positive charged residue of substrate. Thus, Arginine derivatives are used as substrates to study trypsin activity. So far, many mammalian trypsin has been studied, but fish trypsin possesses a high catalytic activity even at low temperature. This unique property makes their applicability in many industries and biotechnology Victor dos Santos [2]. Moreover, fish visceral waste can be valorized optimally by obtaining visceral protease trypsin Aissaoui [3]. The economic importance of waste derived trypsin can be assessed by the fact that the waste can be utilized in a cost-effective manner Das et al. [4]. According to Klomklao & Benjakul fish visceral waste produced upon processing of fish, comprises around 20-30% of the fish weight.

Fish trypsin from diverse habitats has been isolated including marine, freshwater and gulf. Wang et al. [5] have purified and characterized trypsin from hybrid tilapia Oreochromis niloticus x O. aureus. Trypsin exclusively from fish waste has been isolated by Jellouli et al. [6]; Silva et al. [7]; Unajak et al. [8]; Costa et al. [9]; Blanco et al. [10]; Khandagale et al. [11]. The application of fish waste-derived trypsin in industries demands its compatibility with commercial (bovine) trypsin. This existing source carries a high risk of bovine spongiform encephalopathy (BSE). In this context, fish waste-derived trypsin can prove to be a safer alternative. Thus, scientists compare trypsin for different sources on the basis of activity, amino acid sequence and structure Toyota et al. [12]. The present study focused upon isolating and purifying trypsin from visceral waste of freshwater fish Catla catla, one of the most preferred Indian major carps. The purified enzyme was characterized by studying enzyme kinetics and measuring kinetic parameters. Also, assessment of its potential industrial application was done using a high throughput proteomic technique O-HRLCMS. This as a 'bottom-up' approach, used to identify the MS spectra of the peptides by generating a sequence coverage map. Based upon this map, the similarity with commercial trypsin was established. Besides, using bioinformatics tool, BLAST, similarity with other fish trypsin was also recognized.

Materials and Methods

Sampling

Sampling was performed at 4 different local fish markets of Bhopal (Banjari fish market, Bittan market area, Kotra Sultanabad fish market and Shahpura fish market). The fishes identified as Catla catla using an online database Fish Base. Adult healthy fishes weighing 2Kg (±0.5) and were collected during post-monsoon and pre-monsoon seasons. Their visceral waste liver and intestine (Figure 1) were without delay kept in an ice-box and then transferred to Molecular Biology Laboratory, Department of Biotechnology, Barkatullah University, Bhopal. From the waste 100g liver and 100g intestine (Axis LC/GC weighing balance) was excised, washed with 0.8% saline, labeled and stored at -20˚C for further use.

Figure 1: Viscera.

Figure 2: Catla catla (Catla).

Preparation of crude enzyme extract

Using the method of Simpson & Haard [13], A 10% homogenate of liver and intestine was prepared in extraction buffer (1mM Tris-HCl and 1mM $CaCl_2$; pH 8). The homogenate was agitated (200rpm; 4°C; 30min), followed by centrifugation (11,400g; 4°C; 30min). The supernatant was collected and labeled as Crude Enzyme Extract. Protein content was determined by Lowry's method Lowry et al. [14] using standard bovine serum albumin (BSA). The extract was stored at -20˚C in deep freezer.

Clarification of the crude enzyme extract

The proteins in the crude enzyme extract were precipitated using TCA: Acetone (1:3), ice-chilled acetone (1:3), and salt ammonium sulfate (30% and 80% saturation). After the addition of each precipitant, incubation was provided for 15min at -20˚C and later centrifuged (6,000g; 4°C; 30min). Only the pellet was retained. The ammonium sulfate soluble fraction was dialyzed overnight using tubing made up of eggshell membrane against the extraction buffer at 4°C.

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Purification of trypsin using gel filtration chromatography

Protein was purified using DEAE-cellulose column chromatography. A column of dimensions 0.5 x 5.5cm was prepared in which gel slurry prepared in extraction buffer was added. The column was equilibrated with the extraction buffer, and the sample was applied @ 0.25mlmin^{-1}. The buffer was used to elute out all the fractions which were then collected till no absorbance was detected at 280nm. The fraction giving highest peak was selected.

Assay of activity of trypsin

The capacity of purified trypsin to degrade amide substrate BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) into p-nitroaniline was determined according to the method of Erlanger [15]. For this, 100µl of enzyme sample was added to an aliquot of BAPNA in tris buffer (pH 8.2). The release of p-nitroaniline (yellow color) was measured at 410nm after 10 second intervals for 3 min. by spectrophotometer. One unit of amidase activity is defined as 1µmol product formed per min at 25°C.

$$U = \frac{\Delta A\ 410nm/(\min) \times f \times V}{\eta \times l \times v}$$

Where,

U = amidase activity units

f = conversion factor (10^6)

η =extinction coefficient of p-nitroaniline *i.e.* 8800

$\Delta A\ 410nm/(\min)$ = absorbance change at 410nm per min

V = reaction volume (ml)

l = path length (cm)

v = volume of enzyme (ml)

Enzyme kinetics

Determination of Michaelis-Menten constant (Km) and maximum velocity of the reaction (Vmax) was done by constructing Lineweaver-Burk double reciprocal plot using GraphPad Prism software version8. From these values, turnover number (kcat) and enzyme efficiency were also calculated (kcat/Km).

Determination of pH and temperature of trypsin

Buffers of varying pH (0.1 M citrate-NaOH: pH 4.0 and 6.0; 0.1M Tris-HCl: pH 7.0, 8.0 and 8.5; 0.1M glycine-NaOH: pH 10) were prepared. An equal volume (50µl) of both enzyme sample and substrate were added to 900µl of buffer solution and incubated for 5min. at 37°C. Henceforth, to stop the reaction 1ml 0.1M KOH was added and the yellow color produced was measured at 405nm. The activity was plotted as a function of pH Castillo Yanez [16]. Similarly, the temperature dependency of the enzyme was calculated within a range of 20°C to 70°C. The rate of BAPNA hydrolysis by the enzyme was measured as change in absorbance at 480nm min^{-1} Castillo Yanez [16].

Determination of molecular weight

The molecular weight of the enzyme was found by SDS polyacrylamide gel electrophoresis (Laemmli, 1970). The method was modified by using 6% resolving gel and 4% stacking gel.

Peptide fingerprinting and mass spectrometry

Peptides of the enzyme sample were obtained by self-cleavage in the presence of 50mM ammonium bicarbonate buffer at 37°C for 12hr. Later, desalting was done using Zip-Tip C18 (Millipore Co., Billerica, MA, USA). Using α-cyano-4-hydroxy-cinnamic acid spotting was performed.

Statistical analysis

The experiments were performed in triplicates and their values represented as mean ± standard deviation. Using analysis of variance (ANOVA) the data was analyzed and only those values significant above confidence level 95% ($p<0.05$) was accepted.

Result and Discussion

Determination of protein content in crude enzyme extract

Table 1: Seasonal variation of protein content in crude extracts.

S. No.	Sample	Protein Content (mg ml^{-1})	
		Post-Monsoon	Pre-Monsoon
1.	CL-I	165.43 (± 0.01)	160.45 (± 0.00)
2.	CI-I	138.86 (± 0.01)	138.00 (± 0.01)
3.	CL-II	147.98 (± 0.02)	146.66 (± 0.02)
4.	CI-II	151.11 (± 0.01)	150.00 (± 0.02)
5.	CL-III	140.96 (± 0.02)	138.76 (± 0.02)
6.	CI-III	141.12 (± 0.02)	140.00 (± 0.02)
7.	CL-IV	175.32 (± 0.01)	171.76 (± 0.01)
8.	CI-IV	145.22 (± 0.01)	140.78 (± 0.00)

The protein content was analyzed (Table 1) and it was found that the values were consistently higher in post-monsoon samples than in pre-monsoon samples. The physiological factors such as spawning, feeding, and maturation contribute towards seasonal variation in protein content Ravichandran [17]. Amongst all, the hepatic extracts exhibited a higher protein value which may be due to inclination of liver towards metabolic processes. Only in site 2 the intestinal extract exhibited 2% increase than liver. This suggests site 2 to harbor fishes from lotic habitat Tiwari & Pandey [18]. The results were in accordance with the findings of Pilla [19] while working on viscera of Lutjaus johni. Also, Prasad [20] found similar results in marine fishes Gazza achlamys and Ariomma indica.

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Clarification of crude extract

For precipitation of the proteins present in the crude enzyme extract, different chemicals were used. Table 2 & 3 resp. depicts the protein content obtained and weight of pellet obtained in each case. All the precipitating agents were found resourceful as a significant amount of pellet (Table 2) was obtained in each case. Although, ammonium sulfate precipitation method resulted into highest pellet weight (591.01mg) among all, yet it was found with lower protein content (111.39mg ml^{-1} as highest value) than cold acetone precipitation method (133.25mg ml^{-1} as lowest value) (Table 3). This may be because of presence of salt particles molecules along with pellet by forming hydrogen bond with precipitated protein molecules as emphasized by Purwanto [21]; Lamas [22] have also reported cold acetone precipitation method effective at initial stage of purification of trypsin from Merluccius hubbsi visceral waste.

Table 2: Measurement of pellet weight and protein precipitated by different precipitating agents.

S. No.	Method	Season	CL-I		CI-II		CL-IV	
			Protein (mg ml^{-1})	Pellet Weight (mg)	Protein (mg ml^{-1})	Pellet Weight (mg)	Protein (mg ml^{-1})	Pellet Weight (mg)
1	Cold acetone	Post-monsoon	150.33 (±0.01)	27.65 (±0.01)	130.99 (±0.01)	25.52 (±0.01)	152.93 (±0.01)	28.80 (±0.01)
		Pre-monsoon	147.99 (±0.01)	22.54 (±0.01)	130.01 (±0.01)	20.76 (±0.01)	140.90 (±0.01)	25.09 (±0.01)
2	$(NH_4)_2SO_4$ (30% saturation)	Post-monsoon	99.90 (±0.01)	850.73 (±0.02)	115.49 (±0.01)	475.00 (±0.01)	100.26 (±0.01)	861.23 (±0.02)
		Pre-monsoon	92.23 (±0.02)	845 (±0.01)	113.43 (±0.02)	465.00 (±0.02)	91.56 (±0.02)	851.67 (±0.02)
3	$(NH_4)_2SO_4$ (80% saturation)	Post-monsoon	110.98 (±0.01)	261.08 (±0.01)	103.00 (±0.01)	237.38 (±0.02)	100.39 (±0.01)	242.23 (±0.01)
		Pre-monsoon	96.04 (±0.02)	250.01 (±0.01)	82.69 (±0.02)	220.75 (±0.01)	95.99 (±0.01)	233.99 (±0.02)
4	TCA: Acetone	Post-monsoon	85.59 (±0.01)	38.9 (±0.00)	92.32 (±0.01)	33.70 (±0.02)	73.26 (±0.01)	41 (±0.00)
		Pre-monsoon	74.47 (±0.01)	29.02 (±0.00)	82.00 (±0.02)	24.56 (±0.02)	66.78 (±0.01)	32.89 (±0.01)

Table 3: Determination of Peaks Obtained from Digest of CL-IV Fraction.

S. No.	Retention Time (min)	Position	Sequence	m/z value
1.	13.93	5-14	IEVRLGEHNI	1180.489
2.	14.01	117-124	TMFCAGYL	905.386
3.	14.07	120-131	CAGYLEGGKDSC	602.315
4.	24.93	49-56	LSTPASLN	802.43
5.	25.47	137-140	GPVV	371.227
6.	27.85	14-19	IVVNEG	630.346
7.	41.63	34-100	SYDSYTLDSDVMVIKLSTPASLNQYVQPISLPSGCAAAGTKCSVTGWGN-TM SPTADSDKLQCLEIPI	1761.602

Purification of trypsin by gel filtration chromatography

On performing Gel Filtration Chromatography, the fraction exhibiting highest absorbance was compared with the standard and crude extract. Appearance of peak around 280nm in the crude extract indicates presence of trypsin. However, the broader peaks indicate presence of other proteins (Figure 3). A combination of Sephadex $G^{-1}00$ and DEAE-Cellulose column chromatography has been applied by Geethanjali & Subash 2018, for purifying Labeo rohita visceral protease to obtain 13.40-fold purity.

Kinetic characteristics of trypsin in chromatographic fraction of CL-IV

The kinetic characteristics of enzyme include study of certain parameters such as activity, specific activity, optimum pH and temperature, efficiency and turn over number. In the present study, activity and specific activity of trypsin for BAPNA (N-α-benzoyl-DL-arginine p- nitroanilide) hydrolysis was calculated as 15.54U ml^{-1} and 144Umg^{-1}, resp. Using GraphPad Prism software version8, a Lineweaver-Burke plot (Figure 4) was constructed to

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find the value of Km (2.47mM), Vmax (0.40µm^{-1} min^{-1}) and kcat (3.8 s^{-1}). These results were consistent with those of Duarte et al. 2013 while working on hepatic trypsin activity of herbivore and omnivore Siluriformes fish species. However, a correlation between digestive enzyme activity and feeding habit of fish is still not clear.

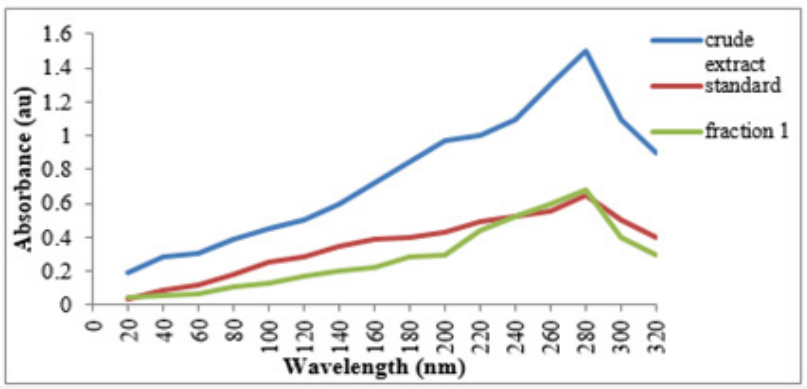

Figure 3: Absorption spectra of crude extract, Standard/commercial trypsin, and Fraction1= chromatographic fraction1

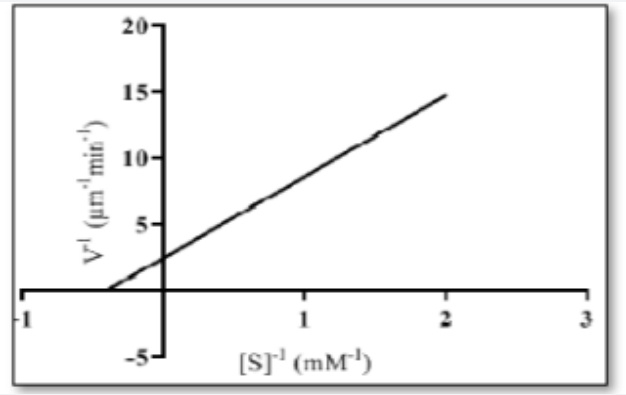

Figure 4: Lineweaver-Burk Plot of fraction CL-IV.

Determination of optimum temperature

The enzyme sample was incubated with the substrate at different temperatures (20°C -70°C) and activity was studied (Figure 6). Activity of trypsin gradually increased with increasing temperature, reached maximum (15.41U ml^{-1}) at a temperature of 40ºC and then decreased. The residual activity in CL-IV at temperatures 50ºC and 60ºC was 58.5% and 46.3% resp. but 13.1% even at 70ºC. Thus, the optimum temperature was 40ºC and a residual activity at higher temperatures was also observed. These findings were in line with the findings of Khangembam [23]. Comparatively, a lesser residual activity (46.3%) at 60 ºC but an improved activity (13.1%) at 70ºC was observed. Such an observation highlights over the possibility of thermostable nature of the fish enzyme which may be due to presence of Ca^{2}+ used in extraction buffer Bougatef [24]. Such enzymes are useful as meat tenderizing agent and detergents which requires higher working temperature (upto 70°C). However, fish trypsin to be applied in detergent industries, requires prior studies of detergent compatibility Tavano [25].

Determination of molecular weight

The purified enzyme exhibited a single band on SDS-PAGE at 24KDa (Plate 1). Fish trypsin has been reported with a molecular weight in the range 20-30 KDa Khandagale [26] which depends upon their varying habitat and geographical conditions. Analysis of Peptides by Orbitrap High Resolution Liquid Chromatography Mass Spectrometry (O-HRLCMS). For a comprehensive study of the purified protein, peptide analysis was performed using Orbitrap High Resolution Liquid Chromatography Mass Spectrometry. A Total Ion Current Chromatogram (TIC) was obtained (Figure 7) and the peptide sequences were analyzed in terms of their m/z value (Table 3). An examination of TIC by MASCOT search engine the purified enzyme exhibited 19.3% resemblance with commercial bovine trypsin. The peptide sequences were aligned (Figure 8) and compared with the sequences of other fishes using BLAST

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(Table 4). Similarly, based on the peptide sequences obtained from LC-MS, Kumazawa [27] identified the multiple sources of animal glue/collagen. They detected collagen from eight different animal sources. In the same way, He et al. [28] also demonstrated the potential of LC/MS technique in identifying common vitellogenin protein form multiple fish species [29].

Table 4: The comparison of percentage identity of the sequence of peptides with other fishes resulted from Blast.

S. No.	Sequence Fragment	Position	BLAST Result				
			Organism	Max Score	Query Coverage	Identity	Accession Number
1	EGTEQFI	18-24	*Lutjanus purpereus*	26.1	100%	100%	AMW07444.1
			Papio anubis	26.1	100%	100%	XP017811734.1
			Anabas testidineus	26.1	100%	100%	XP026226638.1
			Scleropages formosus	26.1	100%	100%	XP018587676.1
2	YPGMIT FCAGY	110-123	*Cyprinus carpio*	52.8	100%	100%	BAL04386.1
			Labeo rohita	52.8	100%	100%	AHY00277.1
			Carrasius auratus	52.8	100%	100%	XP026104836.1
			Danio rerio	52.8	100%	100%	NP955899.2
3	MFCAGY LE	118-125	*Amphiprion ocellaris*	31.6	100%	100%	XP023119402.1
			Salmo salar	31.6	100%	100%	XP014057038.1
4	NSYPGM ITNTMF CAGY	108-123	*Cyprinus carpio*	58.7	100%	100%	BAL04386.1
			Labeo rohita	58..7	100%	100%	AHY00277.1
			Carrasius auratus	58.7	100%	100%	XP026104836.1
			Salmo salar	58.7	100%	100%	XP014010924.1

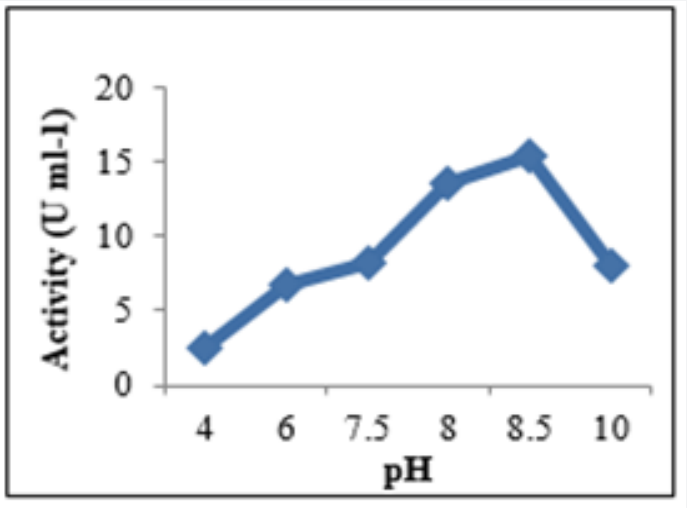

Figure 5: Showing optimum pH for CL-IV.

Conclusion

The kinetic parameters and results of LCMS relate the isolated trypsin with that of other fish and mammalian trypsin. The effect of pH and temperature highlights the potential application of this enzyme in detergent and in the food industry. The industrial detergent enzyme market has been expected to exhibit a growth rate of 11.3%. Thus, a more beneficial and productive use of fish processing waste could be made in an eco-friendly manner. In relevance of the study, the fish processing industries could be suggested to set up a unit in the near vicinity of fish catch so that the waste can be quickly collected and processed into industrially important enzyme.

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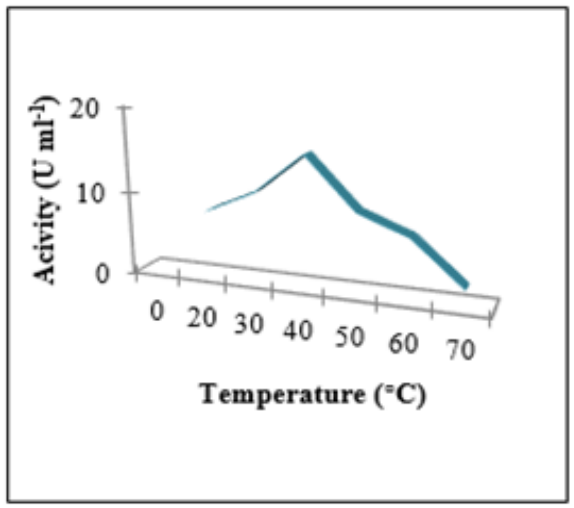

Figure 6: Showing optimum temperature for CL-IV.

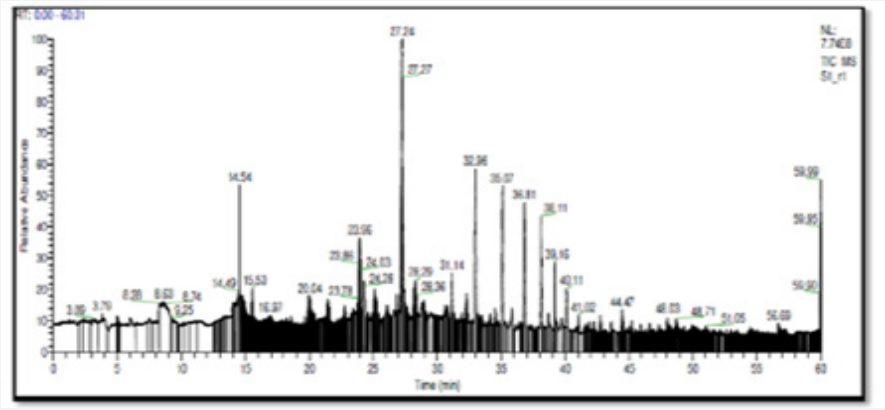

Figure 7: Total Ion Current Chromatogram of CL-IV.

Protein	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:Trypsin	7	0.0%	19.3%	100.00%
Unidentified	35032	100.0%		

MS Signal Threshold = 5.2e+002

Trypsin

Y K S R I E V R L G E H N I V V N E G T E Q F I N S A K V I R H P S Y D S Y T L D S D V M V I K L S
40.6
T P A S L N Q Y V Q P I S L P S G C A A A G T K C S V T G W G N T M S P T A D S D K L Q C L E I P I
60.0
L S D T D C S N S Y P G M I T N T M F C A G Y L E G G K D S C Q G D S G G P V V R N G Q L
34.7
28.2

Figure 8: Sequence Coverage Map of digest of CL-IV fraction.

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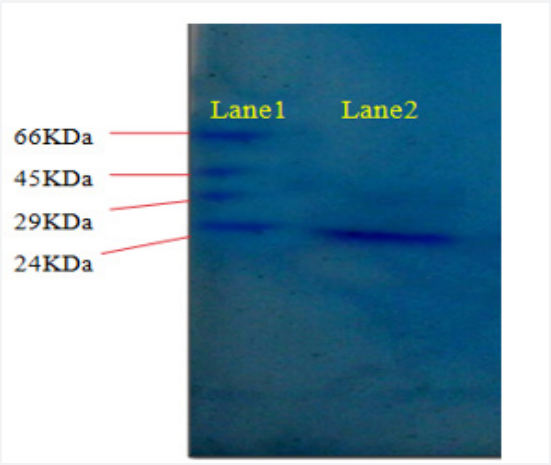

Plate 1: Electrophoretogram of CL-IV (lane2) running along with protein marker (lane1).

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