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Enzymatic Extraction and Characterization of Bioactive Compounds of Fish Visceral Hydrolysate of Labeorohita

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Abstract
Fish viscera, which is usually considered a waste product, has been found to be a valuable source of proteins, peptides, and amino acids that can be used in various applications. In this paper we focus on the application of fish visceral hydrolysate from Labeorohita in food and pharmaceutical industries and other industries. The hydrolysate was obtained by enzymatic hydrolysis using alcalase and protease enzymes. The resulting hydrolysate had a high protein content and antioxidant activity. In the food industry, the hydrolysate was used as a food ingredient to enhance the nutritional value and functional properties of food products. The hydrolysate was also found to have antimicrobial properties, which can be utilized as a natural preservative in food products. In the pharmaceutical industry, the hydrolysate was tested for its potential use in wound healing and as an anticoagulant. The results showed that the hydrolysate had a significant effect on wound healing and could be used as a potential natural anticoagulant. In conclusion, fish viscera hydrolysate from Labeorohita has great potential as a source of bioactive compounds that can be used in various applications in food and pharmaceutical and other industries.
Keywords: *Fish visceral hydrolysate, Labeo rohita, protease enzymes, bioactive compounds*

INTRODUCTION

Fish waste contains a variety of useful nutrients, including proteins, amino acids, bioactive peptides, collagen, gelatin, calcium, enzymes, precious oils, minerals, lipids, colors, and tastes. Value-added goods can be produced using biotechnological methods, lowering the environmental waste burden. There are several of them, but enzymes are the most crucial. This includes collagen, fish hydrolysate, fish oil, gelatin, lipases, lysozyme, proteases, phosphatases, and esterase, among others[1].

In comparison to synthetic or inorganic catalysts, enzymes have a remarkable catalytic potential and are substrate-specific[2]. Enzymes are extensively investigated from an industrial perspective aside from the fundamental approach. The integration of enzyme technology with scientific progress and translation of laboratory results into practical technology at a commercial scale have lead to betterment of the living standard[3]. Moreover, valorization of fish visceral waste into industrially and commercially applicable enzymes has been emphasized as a promising alternative for environmental and economic advantage and contributes towards sustainable development[4].

Fish waste is a good source of digestive enzymes. As these poikilotherms have varied feeding habits and habitat preference, their digestive enzymes are diversified (Yang, 2009)[5]. Fish age and developmental stage influences their enzyme activity. Cold water inhabiting fish species have enzymes with low activation energy and temperature optima, higher thermal instability at alkaline pH. On the other hand, warm water inhabiting fish species exhibit properties opposite to their counterpart. Similarly, marine water fish enzymes exhibit certain unique properties[6].

Among the visceral enzymes, proteases contribute towards 60-65% of global fish market. These are the enzymes that break down the peptide bonds that connect the amino acids in a protein's polypeptide chain. Proteases have a significant role in biological processes and the pathogen life cycle, which leads to their use in the biotech and research sectors[7]. This includes peptide synthesis, production of Klenow fragment, digestion of protein to obtain nucleic acids from cell extract, digestion of protein sample to obtain a peptide fingerprint map, recombinant protein from fusion protein, tissue dissociation during cell culture and preparation of recombinant antibody fragment[8]. The growth of proteases during last two decades has been observed in baking industry, animal feed industry, organic synthesis and paper industry[9]. Proteases include chymotrypsin, pepsin, collagenases and calpains, lipase, cathepsin and trypsin is the most important one. Due to its ability to remain active throughout a wide pH and temperature range, even at lower concentrations, trypsin has found use in both industrial and non-industrial settings. Trypsin is applied in food industries for- cheese and yogurt making, meat tenderization, hydrolysate production and manufacturing of savory products. Trypsin is required as a formulating

agent in detergent industries and in laundries. In leather industries, trypsin is required as a dehairing and bating (softening) agent in leather processing[10] . The applications in pharmaceutics include: treatment of inflammatory conditions, clotting and gastrointestinal disorders, regulation of complement system and apoptotic pathway, and production of contact lens cleaning formulations. Other industrial application include- wastewater treatment, gelatin hydrolysis, fish protein hydrolysate production, extraction of chitin from crustacean waste, extraction of rice starch, silk degumming, silver recovery from used photographic plate, isolation of cells and tissue dissociation for animal cell culture[11]. A new emerging application of proteases is conversion of waste into useful biomass. The use of micro-organisms for this purpose is trending which is depended upon costly fermentation technology. Thus, cost-effective and more economic alternatives are needed to meet the ever-growing demand for this enzyme. Generally, enzymes are derived from plant, animal and microbial sources. Although extraction from microbes is simplest, easiest, cost-effective and easy to scale-up, yet the cost of approval by regulatory authorities is very high and subtle differences in enzyme structure makes them less suitable for applications in industries[12]. Fish derived enzymes are not as popular as enzymes from other sources as they are not as well investigated, the raw material may only be seasonally available and they are considered unattractive due to preconceived ideas about odors. But fish waste can prove to be a new source of enzymes[13].

RESEARCH METHODOLOGY

❖ Fish visceral enzymes

Fish waste has been proved as a good source of enzymes such as lipases, lysozyme, proteases, amylases, phosphatases, and esterases etc. The enzyme amylase participates in carbohydrate metabolism and has been employed in industries as food additive. In brewing industries, it is used in production of bread, glucose and fructose syrups, alcoholic beverages, sweeteners, and as digestive aid. Amylase is also applicable for fuel production from ethanol, and in paper and detergent industry. The property of amylase of increasing drug release rate has enabled it useful in molecular biology to study gene regulatory elements and gene expression. Studies on fish amylase extraction are limited[14]. Some of these include isolation from mackerel (Park et al., 2008), mackerel, menhaden, scup, sea robin, puffer and toad fish (Chesley, 1934), sea bream, turbot and red fish, parrot fish and stargazer[15].

The enzyme which catalyzes hydrolysis of triacylglycerol into free fatty acids and glycerol thereby metabolizing lipids, are called as lipases. The biochemical properties of lipases have made it suitable for industrial purposes. It is useful in detergent formulation, cheese-making, fermented sausage production, removing pitch from pulp for paper production, synthesizing organic compounds, cosmetics, biodiesel production, as biosensor to detect contaminants/pesticides/toxins.

Proteases are the enzymes which break the peptide bond present in proteins to form amino acids. Proteases rank first in industrial demand among all other enzymes. Proteases dominate among industrial enzymes because of their unique properties. They selectively modify proteins by limited proteolysis; can be used in one or other form in medical therapy, as reagents in laboratories and industries. Their fastest growth in previous decade has been observed in baking and animal feed industries[16].

The anti-inflammatory property of chymotrypsin makes it applicable in pharmaceutical industries. As the enzyme contributes to proper digestion, it acts as a chemotherapeutic in dyspepsia and anorexia. Chymotrypsin cleaves the neurotoxins and cytotoxins present in snake venom thereby preventing cell death by snake-bite[17]. Also, the enzyme has been found effective in treatment of infertility because of its ability to shorten semen liquefaction time without affecting sperm motility. The enzyme enhances decontamination ability of detergents and works under mild conditions, i.e., low temperature and neutral pH so is used as detergent formulation in detergent industries[18].

- **Crude enzyme extract protein determination**

In the post-monsoon and pre-monsoon seasons, 120 fish (Catlacatla and Labeorohita) were collected from various locations. To remove blood and debris, the viscera (100g liver and 100g intestine) of each species were washed in 0.8% saline before their crude extracts were made. The protein content was examined (Table 1.1), and the extracts with reported values between 150 and 200 mg ml-1 were chosen for additional research. In comparison to pre-monsoon samples, post-monsoon samples consistently had greater protein content. The liver extracts of both species exhibited higher protein content than their respective intestinal extracts. However, in case of Catla samples procured from Site 2, the intestinal extract contained 150 mg ml-1 protein as compared to 146.66 mg ml-1 protein in liver. Although, visceral extracts of Rohu were found to contain more protein than visceral

extracts of Catla, CL-I (165.43 mg ml-1) and CI-II (150.00 mg ml-1) exhibited higher protein values than RL-I (140.23 mg ml-1) and RI-II (140.39 mg ml-1).

Table 1.1: Determination of protein content in crude extracts obtained from liver and intestine during different seasons

S. No.	Sample	Protein content (mg ml^{-1}) Post-monsoon	Pre-monsoon
3.	RL-I	140.23 (± 0.01)	139.78 (± 0.01)
4.	RI-I	139.00 (± 0.01)	140.22 (± 0.01)
7.	RL-II	175.86 (± 0.02)	168.34 (± 0.01)
8.	RI-II	140.39 (± 0.02)	138.23 (± 0.01)
11.	RL-III	184.45 (± 0.02)	178.65 (± 0.00)
12.	RI-III	150.95 (± 0.01)	150.09 (± 0.00)
15.	RL-IV	186.58 (± 0.00)	180.44 (± 0.00)
16.	RI-IV	150.84 (± 0.00)	151.07 (± 0.01)

Mean= ±S. D, n=3

- **Recovery of Trypsin from Crude Extracts**

The enzyme trypsin was recovered from the chosen crude extracts through different steps- precipitation (solvent precipitation and salt fractionation), dialysis and gel-filtration chromatography.

A. Precipitation of Total Proteins Present in Crude Extracts

The protein precipitation was carried out using solvents (cold acetone and TCA: Acetone) and salt (ammonium sulphate) so as to identify samples with highest protein content.

- **Total Protein Content obtained by different Precipitation Methods**

The sample CL-IV collected during post-monsoon season, exhibited highest protein content (152.93 mg ml-1) followed by post-monsoon sample of RL-IV (151 mg ml-1) using cold acetone as precipitating agent. The other samples having protein content in the given range are post-monsoon samples of RL-II (150.49 mg ml- 1) and CL-I (150.33 mg ml-1). Again, the pre-monsoon samples were detected with lower protein values as observed in crude enzyme extracts.

Among all the precipitation methods, solvent precipitation (cold acetone) exhibited more protein in samples than salt fractionation. The cold acetone precipitate of all the samples exhibited higher protein content, followed by TCA: Acetone precipitates, and salt precipitates. Except salt precipitation method, all the methods resulted into higher protein value in liver extracts than in intestinal extracts. Salt precipitation method resulted into high variation in protein content with highest value as 120.62 mg ml-1in RL-IV and lowest value as 165.02 mg ml-1 in RI-III. The highest protein value observed in 80% saturated (NH4)2SO4 fraction was 120.62 mg ml-1 (post-monsoon sample of RL-IV) whereas 115.49 mg ml-1 (in post-monsoon sample of CI-II) in 30% saturated (NH4)2SO4 fraction. The solvent precipitant TCA: Acetone recovered least protein content with 103.96 mg ml-1 as highest and 35.05 mg ml-1 as lowest values. The precipitation methods were also compared on the basis of weight of pellet formed.

Table 1.2 Determination of protein content at different precipitation level with respect to different season

S. No.	Method	Protein content in samples (mg ml^{-1}) Season	RL-IV	RL-III	RL-II	RI-III	RI-IV
1.	Cold acetone	Post- Monsoon	**151.00** (±0.01)	147.49 (±0.01)	**150.33** (±0.01)	140.93 (±0.01)	136.39 (±0.01)
		Pre-Monsoon	148.56 (±0.01)	144.03 (±0.00)	147.99 (±0.01)	137.02 (±0.01)	133.25 (±0.00)

2.	$(NH_4)_2$ SO_4 (30% saturation)	Post- Monsoon Pre- Monsoon	103.43 (±0.01) 110.09 (±0.02)	90.02 (±0.01) 88.30 (±0.01)	99.90 (±0.01) 92.23 (±0.02)	110.30 (±0.01) 99.06 (±0.02)	111.39 (±0.01) 100.22 (±0.02)
3.	$(NH_4)_2$ SO_4 (80% saturation)	Post- Monsoon Pre- Monsoon	120.62 (±0.01) 114.00 (±0.00)	90.39 (±0.01) 84.00 (±0.00)	110.98 (±0.01) 96.04 (±0.02)	117.50 (±0.01) 103.33 (±0.02)	88.50 (±0.01) 70.00 (±0.02)
4.	TCA: Acetone	Post- Monsoon Pre- Monsoon	103.96 (±0.01) 100.00 (±0.00)	55.00 (±0.01) 46.98 (±0.01)	85.59 (±0.01) 74.47 (±0.01)	100.63 (±0.01) 85.34 (±0.01)	50.53 (±0.01) 35.05 (±0.01)

1.1. Measurement of Pellet Weight formed through different Precipitation Methods

It is evident from Table 1.3 that salt precipitation formed more pellet than solvent precipitation. The solvents, formed maximum pellet as 56.60 mg with TCA: Acetone, and 33.68 mg with cold acetone both in post-monsoon sample of RL- III. Salts generally stick with protein molecules hampering correct pellet value thus need to be separated by the method of dialysis.

Table 1.3 Determination of weight of pellet obtained after precipitation from different liver and intestinal samples in different seasons

S. No.	Method	Weight of pellet (mg) Season RL-IV RL-III RL-II RI-III RI-IV					
1.	Cold acetone	Post-Monsoon Pre-Monsoon	32.00 (±0.01) 30.34 (±0.01)	33.68 (±0.01) 31.65 (±0.01)	28.99 (±0.01) 25.78 (±0.01)	26.85 (±0.01) 22.46 (±0.01)	26.90 (±0.01) 23.76 (±0.01)
2.	$(NH_4)_2SO_4$ (30% saturation)	Post-Monsoon Pre-Monsoon	392.00 (±0.02) 389.72 (±0.02)	470.76 (±0.01) 465.99 (±0.02)	390.43 (±0.03) 384.09 (±0.02)	590.81 (±0.02) 581.90 (±0.03)	591.01 (±0.02) 582.79 (±0.01)
3.	$(NH_4)_2SO_4$ (80% saturation)	Post-Monsoon Pre-Monsoon	242.43 (±0.01) 238.22 (±0.02)	260.09 (±0.01) 255.76 (±0.01)	240.54 (±0.02) 231.46 (±0.01)	170.00 (±0.01) 165.02 (±0.01)	188.50 (±0.02) 176.49 (±0.02)
4.	TCA: Acetone	Post-Monsoon Pre-Monsoon	29.53 (±0.01) 20.99 (±0.01)	56.60 (±0.01) 47.66 (±0.02)	54.98 (±0.01) 48.90 (±0.02)	35.87 (±0.02) 27.77 (±0.02)	**24.44** (±0.02) 15.56 (±0.02)

B. Separation of Proteins from Salt Particles using Dialysis

The salt (ammonium sulcate) tends to stick with protein molecules contributing to pellet weight. It is thus separated from protein solution upon dialysis. It also concentrates protein solution. The salt precipitates were dialyzed to remove the salt bound with it and the protein content was compared with that of solvent precipitated methods[19].

The dialyzed fractions were compared in terms of protein content (Fig. 1.1), based on which samples for further purification were selected. Again, the post- monsoon samples exhibited higher protein value than in pre-monsoon samples. The amount of protein was highest in RL-IV (99.57 mg ml-1).The least value of protein was found in pre-monsoon sample of RI-IV (33.02 mg ml-1).

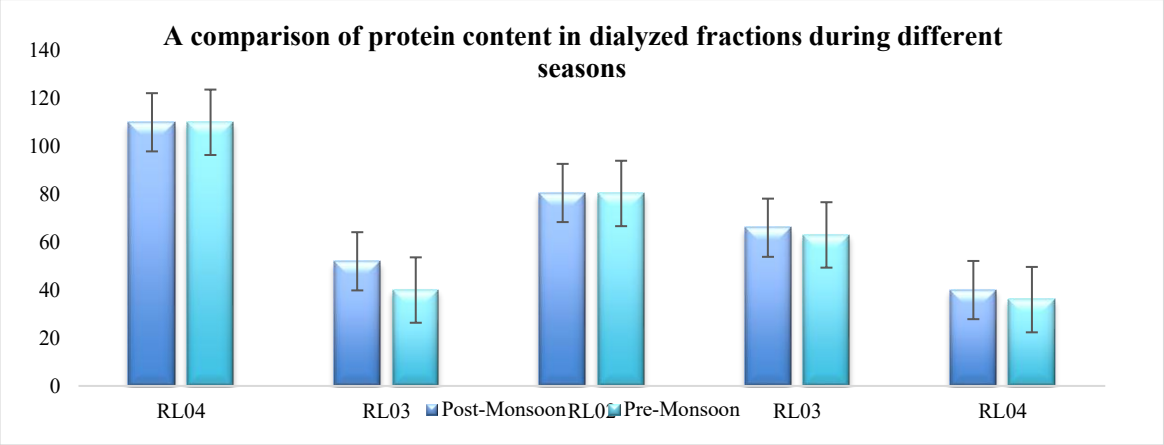

Fig.1.1 A comparison of protein content in dialyzed fractions during different seasons

- **Measurement of Trypsin Activity**

The fractions with protein content within the set range were undergone trypsin activity measurement. The kinetic parameters Km and Vmax, of selected fractions were calculated as shown in Table 1.4. The table indicates highest trypsin activity in RL-IV (182.71 U ml-1), and least in RL-II (51.16 U ml-1). Similar trend was observed in case of specific activity viz. RL-IV (1.21 U ml-1), RL-II (0.34 U ml-1). The affinity of enzyme present in RL-IV towards BAPNA substrate was highest (Km= 2.01 mM). The Vmax value got halved followed by RL-II (0.32 µm-1min-1). The fractions (RL-IV) possessing higher trypsin activity were chosen for further purification through Gel Filtration Chromatography.

Table 1.4 Determination of Kinetic Parameters of Fractions

S. No.	Sample	Activity (U ml^{-1})	Specific activity (U mg^{-1})	Km (mM)	Vmax ($\mu m^{-1}min^{-1}$)
1.	RL-IV	182.711	1.21	2.01	0.29
2.	RL-II	51.166	0.34	2.77	0.32

- **Authentication of Purity of Trypsin**

The SDS-PAGE of precipitates and crude extracts of, RL-IV, RL-II was performed to confirm the purity. Plate 1 clearly indicates presence of different proteins in crude extracts of chosen samples whereas, the electrophoretogram of cold acetone precipitates (Plate 2) depicts single sharp bands indicating precipitation by cold acetone and all the samples exhibited a common band as that of standard trypsin.

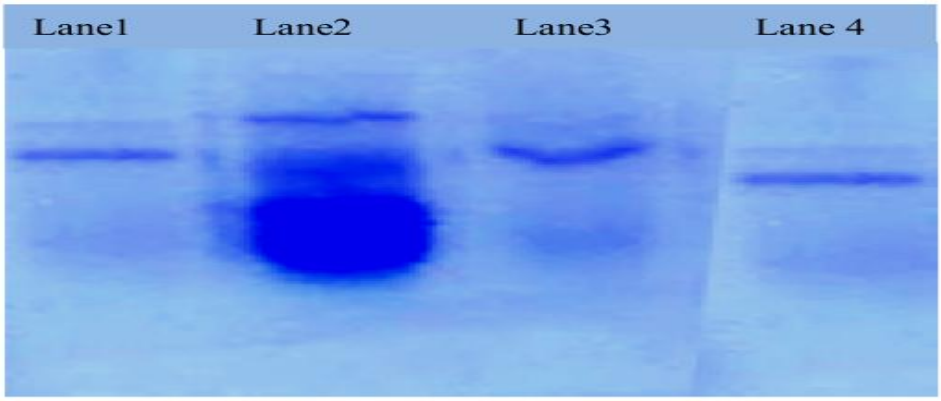

Plate 1: Electrophoretogram of crude extracts of RL-IV (lane1), RL-III (lane2), RL-II (lane3), and RL-I (lane4)

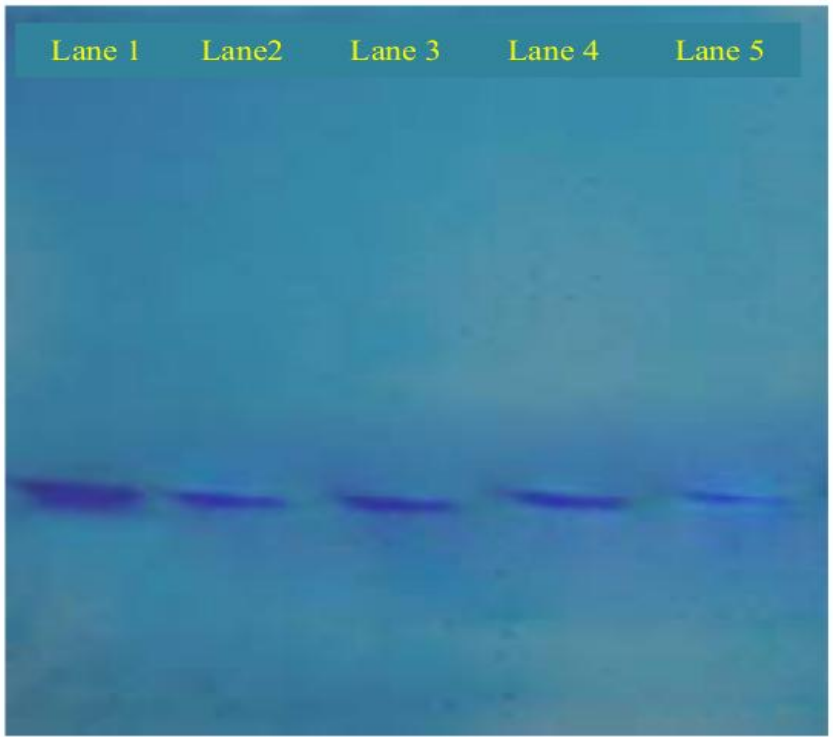

Plate 2: Electrophoretogram of cold acetone precipitates of RL-IV (lane1), RL-IV (lane2), RL-II (lane3), RL-I (lane4) and, standard trypsin (lane5)

Upon electrophoresing ammonium sulphate fractions and TCA: Acetone precipitates (Plate3), high intensity of bands was obtained in ammonium sulphate fractions as compared to latter. A comparison between Plate 2 and Plate 3 emphasized proper precipitation by ammonium sulphate. However, TCA: Acetone precipitates exhibited multiple bands indicating presence of multiple proteins. All samples exhibited a common band as that of standard trypsin[20].

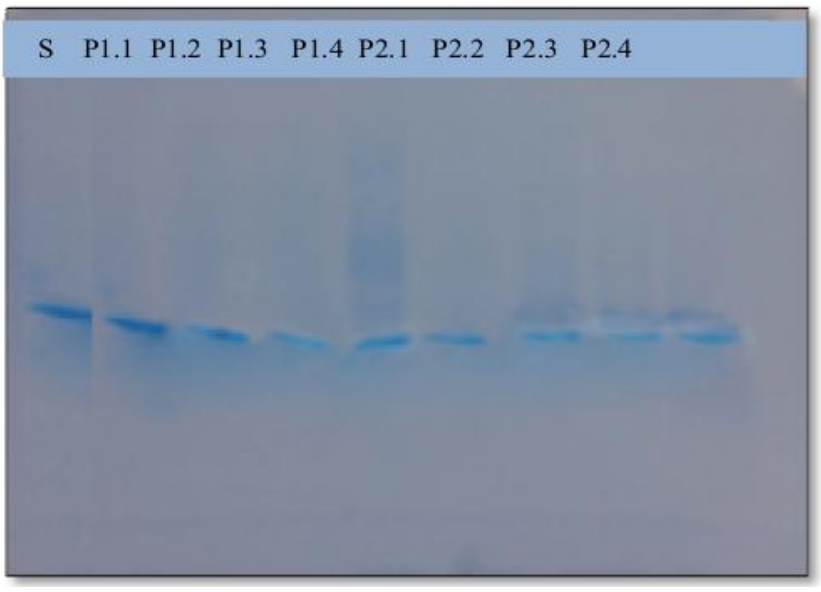

Plate 3: Electrophoretogram of precipitates, P1.1= 30% saturated (NH4)2SO4 fraction RL-IV, P1.2= 30% saturated (NH4)2SO4 fraction RL-IV, P1.3= 30% saturated (NH4)2SO4 fraction RL-II, P1.4= 30% saturated (NH4)2SO4 fraction RL-I, P2.1= TCA: Acetone precipitated RL-IV, P2.2= TCA: Acetone precipitated RL-IV, P2.3= TCA: Acetone precipitated RL-II, and P2.4= TCA: Acetone precipitated RL-I (8 MEGAPIXEL)

C. Gel Filtration Chromatography Trypsin Purification

Gel filtration chromatography is a technique of separation of molecules on the basis of their size, effective for high molecular weight proteins (Castillo-Yanez et al., 2006). The samplesRL-IV was subjected to Gel Filtration chromatography (Fig.4.3) and the fractions were collected. The light absorbance spectrum of samples (Fig.4.5a) gave a bell-shaped curve. The fraction 1 of RL-IV showed highest absorption peaks (0.75 arbitrary unit and 0.80

arbitrary unit resp.) at 280 nm. The appearance of peak around 280nm in the spectra was mainly due to trypsin (Fig. 1.2 and Fig 1.3). The broader peak of crude extract reveals dilution of trypsin than in fraction1 which indicates efficient purification of trypsin.

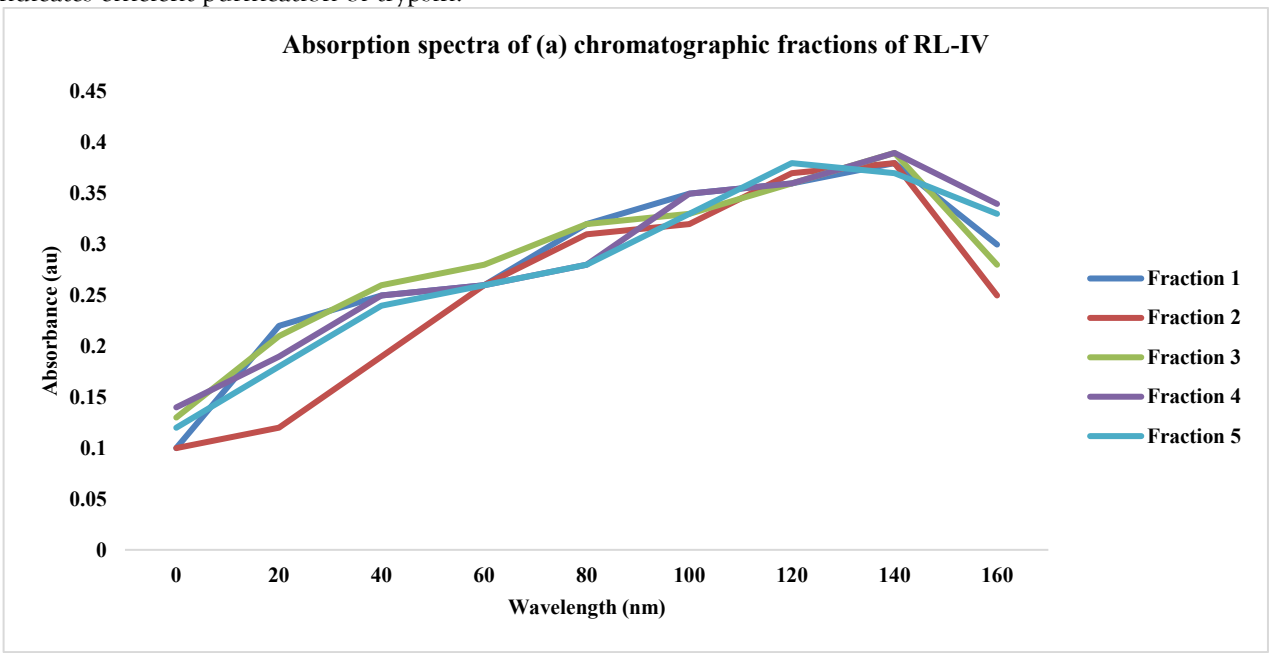

Fig 1.3 (a) chromatographic fractions of RL-IV

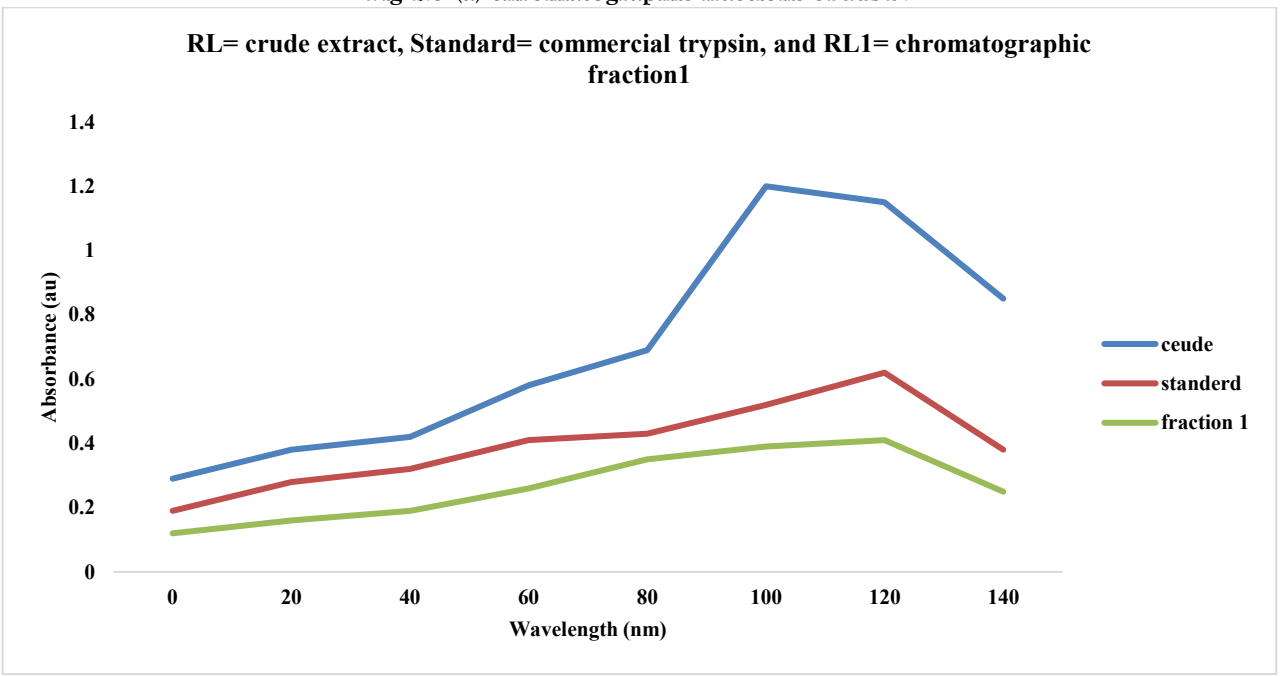

Fig 1.3 Absorption spectra of (a) chromatographic fractions of RL-IV, (b) RL= crude extract, Standard= commercial trypsin, and RL1= chromatographic fraction1

1.3. Characterization of Trypsin in Fractions RL-IV Enzymes

These are primarily characterized on the basis of their rate of catalyzing a reaction. The parameters derived from the study of rate of BAPNA hydrolysis by RL-IV were studied under following heads:

A. Determination of Activity and specific activity of trypsin in Fractions RL-IV

An enzyme's overall catalytic characteristic is explained by its activity, which is a measurement of how much active enzyme is present under certain circumstances. It is calculated as the initial rate of substrate utilization in the absence of products. The activity of trypsin enzyme presents in RL-IV (19.56 U ml-1). The amount of substrate transformed into product per min per mg enzyme under optimum conditions gives the specific activity of that enzyme. It determines the purity of enzyme. The value of specific activity when calculated was found more for RL-IV (179.44 U mg-1)

B.Study of Enzyme Kinetics of Trypsin in Fractions RL-IV

Enzymes are characterized in terms of their substrate affinity and maximum rate of reaction catalyzed by enzyme obtained from Michaelis- Menten equation. As the curve derived from Michaelis- Menten equation does not employs higher variations in substrate concentration, the exact values of the kinetic parameters- Michaelis-Menten constant (Km) and maximum velocity (Vmax) were evaluated from three different types of plots. These were Lineweaver-Burk plot (Fig 4.6), Eadie- Hofstee plot (Fig. 1.4) and Hanes-Woolf plot (Fig. 1.5). At saturating substrate concentration, RL-IV represented rate of reaction (0.40 µm-1min-1). The Km value of RL-IV (2.47 mM) indicates variation in activity of RL-IV with varying substrate concentration.

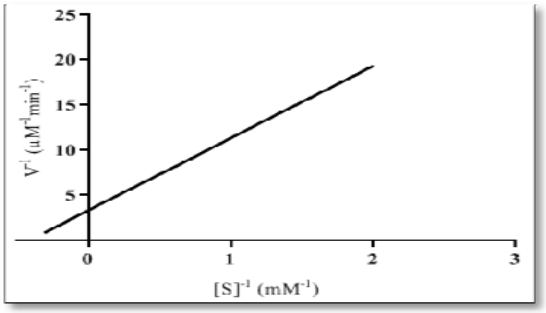

Fig 1.4 Lineweaver-Burk Plots of fractions RL-IV

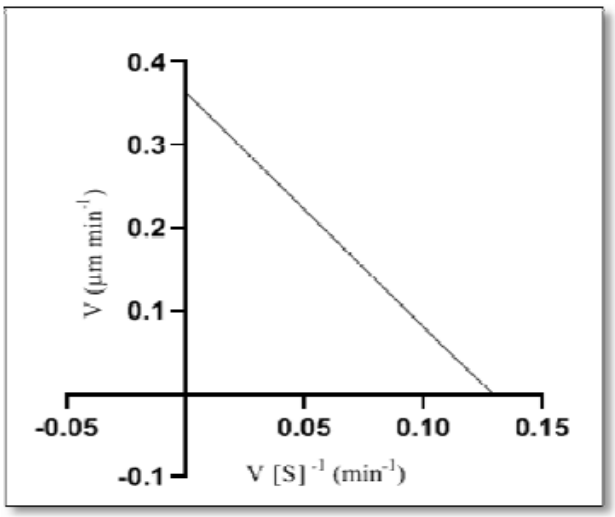

Fig. 1.5 Eadie-Hofstee plots of fractions RL-IV

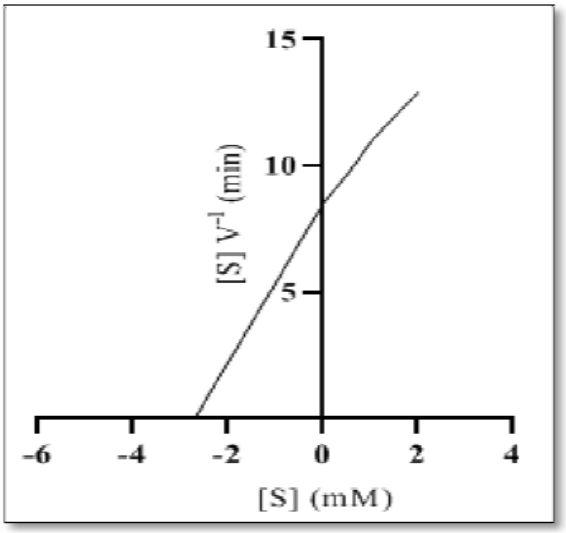

Fig. 1.6 Hanes-Woolf plots of fractions RL-IV

To compare the two enzymes, their turnover number (kcat) and catalytic efficiency (η) was calculated. Table 1.6 shows all the kinetic parameters of purified trypsin. Although CL-IV exhibited high turnover number RL-IV (8.34 s-1 mM-1).

Table 1.5 Determination of Km and Vmax values of Trypsin in Fractions RL-IV by different plots

S. No.	Sample	Parameter	Method		
			Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf
1.	RL-IV	Km (mM) Vmax (μm min^{-1})	2.47 0.40	4.111 0.558	3.158 8.510

Table 1.6 Determination of Kinetic Parameters of Fractions RL-IV

S. No.	Sample	Protein content (mg ml^{-1})	Activity (U ml^{-1})	Specific activity (U mg^{-1})	Efficiency (kcat Km^{-1}) ($s^{-1}mM^{-1}$)	kcat (s^{-1})
1.	RL-IV	0.109	19.56	179.44	8.34	3.5

C. Determination of Optimum pH of Trypsin in Fractions RL-IV

The effect of pH on activity of trypsin was studied at a pH range of 4-10. The pH activity profile of CL-IV is shown in Fig. 1.7a. The activity increased with increase in pH and reached maximum (15.49 Uml-1) at pH 8.5 thereafter showed a sharp decrease (8.01 U ml-1) at pH 10. While trypsin activity in RL-IV was 18.49 U ml-1 at the ideal pH of 8.5, it only retained 49.2% of that activity (9.08 U ml-1) at that pH, compared to 52% in CL-IV (Fig. 1.7b).

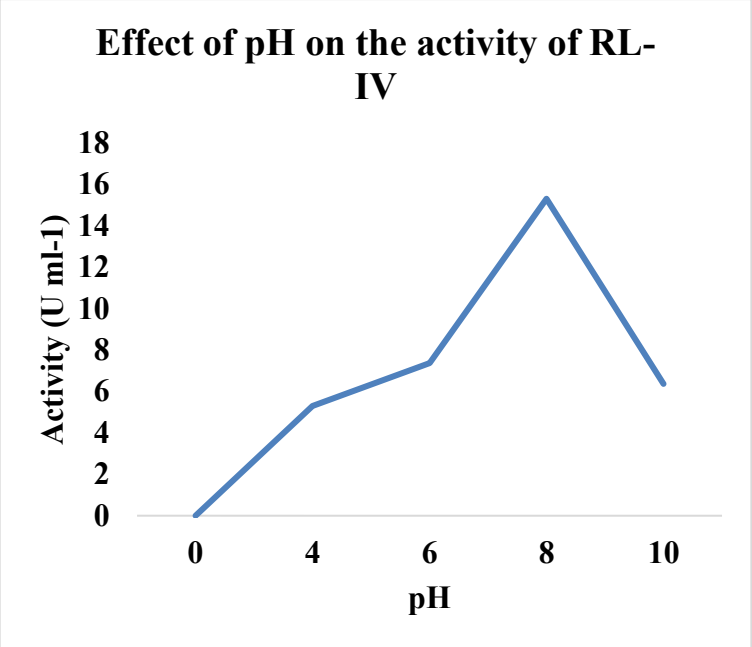

Fig 1.7 Effect of pH on the activity of RL-IV

D. Determination of Optimum Temperature of Trypsin in Fractions RL-IV

The activity of the enzyme was examined throughout a temperature range of 20°C-70°C in order to find the ideal temperature (Fig. 1.8). Trypsin activity in RL-IV steadily rose with rising temperaturepeaked, and then gradually dropped with maximal activity (19.40 U ml-1) at 40°C and a somewhat modest decline in activity thereafter. The enzyme maintained 91.1% of its activity at 50°C, 56.6% at 60°C, but only 9.8% at 70°C.

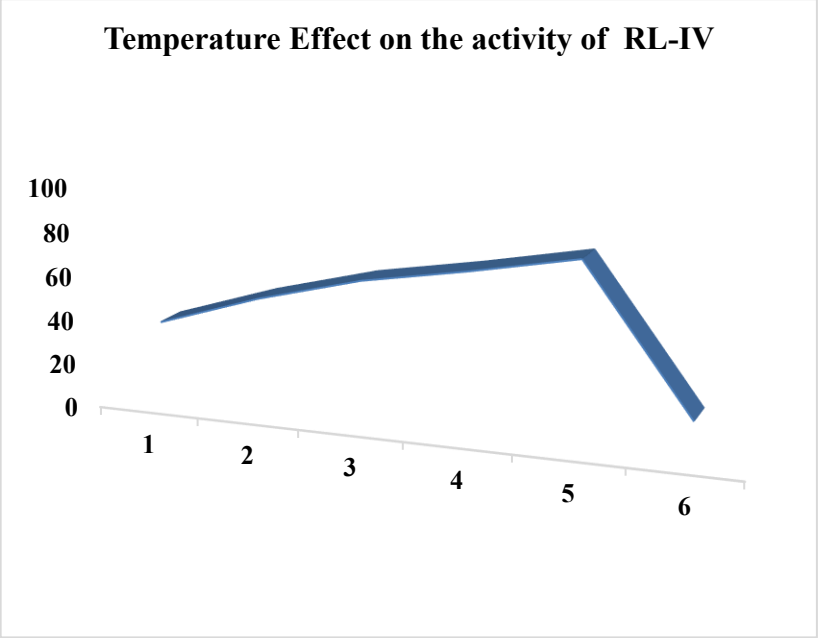

Fig 1.8Temperature Effect on the activity of RL-IV

D. Determination of Molecular weight Trypsin in Fractions RL-IV

With the aim of determining the molecular weight of trypsin in RL-IV, the technique of SDS-PAGE (Fig. 1.9) was performed. The protein marker in lane 1 contains 4 bands with molecular weights- 24 KDa (Bovine pancreatic trypsinogen), 29 KDa (Bovine Erythrocytes carbonic anhydrase), 45 KDa (ovalbumin) and 66 KDa (Bovine Serum Albumin). Based on these bands, the Electrophoretogram (Plate 4) indicates the molecular weight of trypsin in RL-IV to be 24KDa.

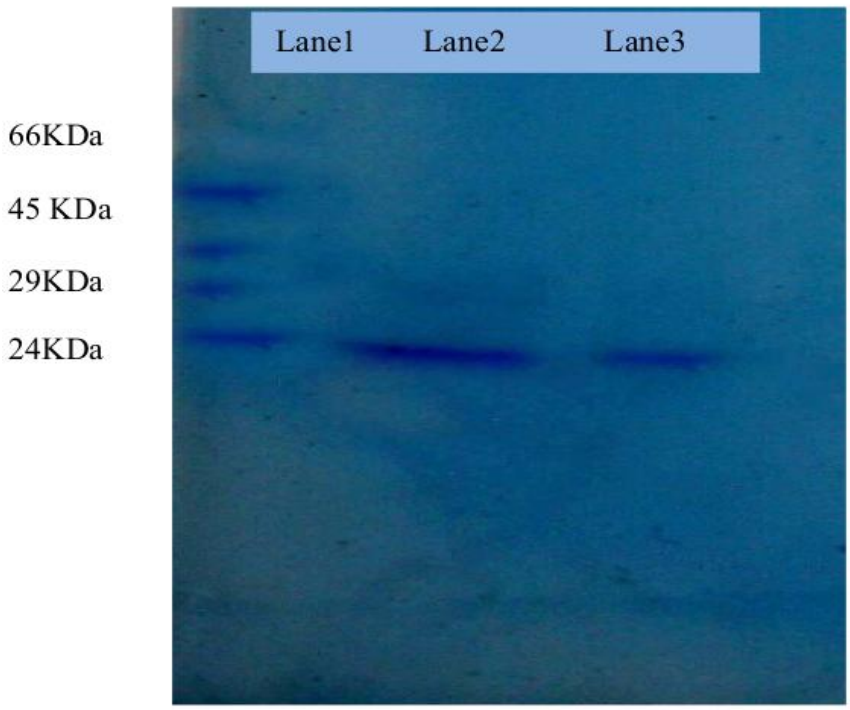

Plate 4: Electrophoretogram of CL-IV (lane2) and RL-IV (lane3) run along with protein marker (lane1)

F. Orbitrap High Resolution Liquid Chromatography Mass Spectrometry (O-HRLCMS) Analysis of Peptides

The peptides that resembled commercial trypsin on the Total Ion Current Chromatogram (TIC) of CL-IV were subjected to spectrophotometer analysis to determine their m/z value and sequence, as indicated in Table.

Fig. 1.9 describes the TIC obtained after proteolytic digestion of RL-IV. The peptides showing similarity with commercial trypsin have been indicated in Table 1.8.

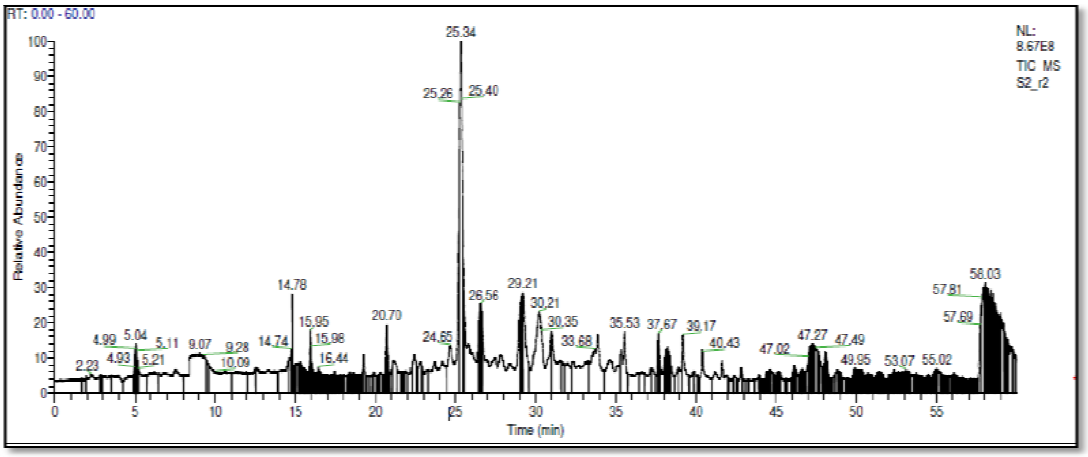

Fig. 1.9 TIC of digest of RL-IV Fraction

Table 1.7Determination of Peaks from RL-IV Fraction Digest

S. No.	Retention time (min)	Position	Sequence	m/z value
1.	13.93	7-36	VRLGEHNIVVNEGTEQFINSAK VIRHPSYD	1712.083
2.	14.01	70-92	AAGTKCSVTGWGNTMSPTADS DK	6763.008
3.	14.07	120-126	CAGYLEG	356.654
4.	24.93	77-88	VTGWGNTMSPTA	1221.562
5.	25.47	106-112	CSNSYPG	724.4
6.	27.85	99-103	PILSD	544.298
7.	41.63	25-32	NSAKVIRH	924.541

The peptide mass spectra were correlated with amino acid sequence from protein sequence database by MASCOT software generating a sequence coverage map (Fig1.10). Both the samples exhibited 19.3% resemblance with commercial trypsin.

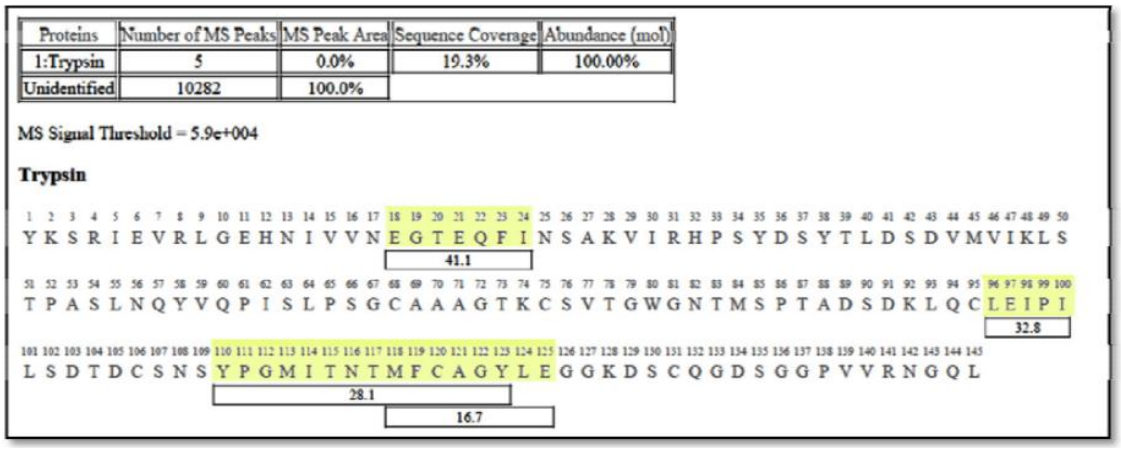

Fig. 1.10Sequence Coverage Map for the RL-IV fraction's digest

As shown in Table 1.8 as the percentage identity of the sequences with other trypsin, the sequences showed similarity with trypsin from other organisms when they were analyzed using BLAST in the protein database (PDB). The samples RL-IV are trypsin, according to the BLAST findings, and they show similarities to commercial bovine trypsin.

Table 1.8 The comparison of percentage identity of the sequence of peptides with other organisms resulted from BLAST

S. No.	Sequence fragment	Position	Organism	BLAST results Max Query score		Identity coverage	Accession number
1.	EGTEQFI	18-24	*Lutjanus purpereus*	26.1	100%	100%	AMW07444.1
			Papioanubis	26.1	100%	100%	XP017811734.1
			Anabas testidineus	26.1	100%	100%	XP026226638.1
			Scleropagesformosus	26.1	100%	100%	XP018587676.1
2.	YPGMIT FCAGY	110-123	*Cyprinus carpio*	52.8	100%	100%	BAL04386.1
			Labeorohita	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	*Amphiprionocellaris*	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
			Labeorohita	58..7	100%	100%	AHY00277.1
			Carrasius auratus	58.7	100%	100%	XP026104836.1
			Salmo salar	58.7	100%	100%	XP014010924.1

G. Qualitative Estimation of Amino Acids in Fractions RL-IV Using Thin Layer Chromatography

TLC was performed to detect amino acids in fractions after hydrolysis (Table 1.9). A Venn diagram shown in Fig. 1.11 clearly depicts that among the total amino acids present in fish protein hydrolysate, three amino acids (Phenylalanine, Alanine and Glycine) were found in sample RL-IV. Alanine was found common in both the samples.

Table 1.9 The detection of Amino acids in hydrolysate of RL-IV based on Rf values

S. No.	Sample	Distance travelled by solvent (cm)	Distance travelled by spot (cm)	Rf value	Standard Rf value	Amino acid

1.	RL-IV	10	3.1	0.31	0.31	A (Ala)
	10	2.0	0.20	0.20	Q (Gln)	

H. Quantitative Estimation of Amino Acids in fractions RL-IV by Ninhydrin Assay

In order to find the concentration of amino acids in samples, Ninhydrin assay was conducted. The number of amino acids in RL-IV respectively, was found to be 130.98 mg 100g -1 and 135.65 mg 100g -1. Fish samples have been reported to contain with amino acid around 140 mg 100g -1 (Ramakrishnan et al., 2013). A relatively less difference in quantity of amino acids was found among the two samples from reported values.

I. Analysis of Trypsin Efficacy in Fractions RL-IV

The efficacy of RL-IV upon KB cell line (Doubling time 50hrs) was assessed in terms of cell viability. At three different time points (10 sec, 15 sec, and 20 sec), the cytotoxic effect of samples at concentrations of 0.01%, 0.1%, and 1% was examined. Commercial (bovine) trypsin (SS) was used as the standard of care. The findings unmistakably show that, when concentration is steadily increased in all treatment groups, cell viability decreases with time.

At various times during the lag phase, Plate 5 shows cell viability at 0.01% concentrations of several trypsinizing agents. The CL-IV maintained a net 15% reduction at all three time points, RL-IV showed variation in reduction of cell viability[38].

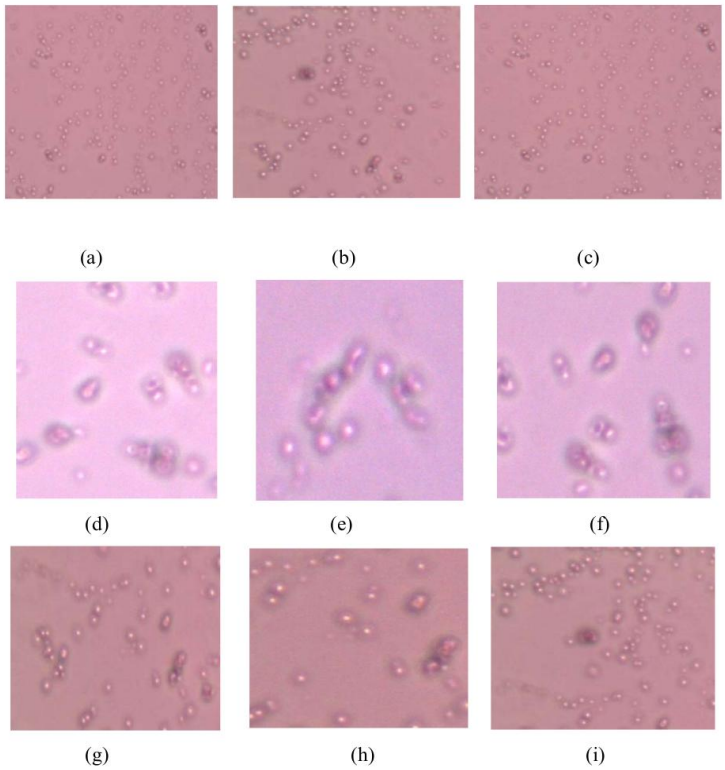

Plate 5: (a) SS for 10 seconds, (b) SS for 15 seconds, (c) SS for 20 seconds, (d) RL-IV for 10 seconds, (e) RL-IV for 15 seconds, (f) RL-IV for 20 seconds, (g) RL-IV for 10 seconds, (h) RL-IV for 15 seconds, and (i) RL-IV for 20 seconds (40X).

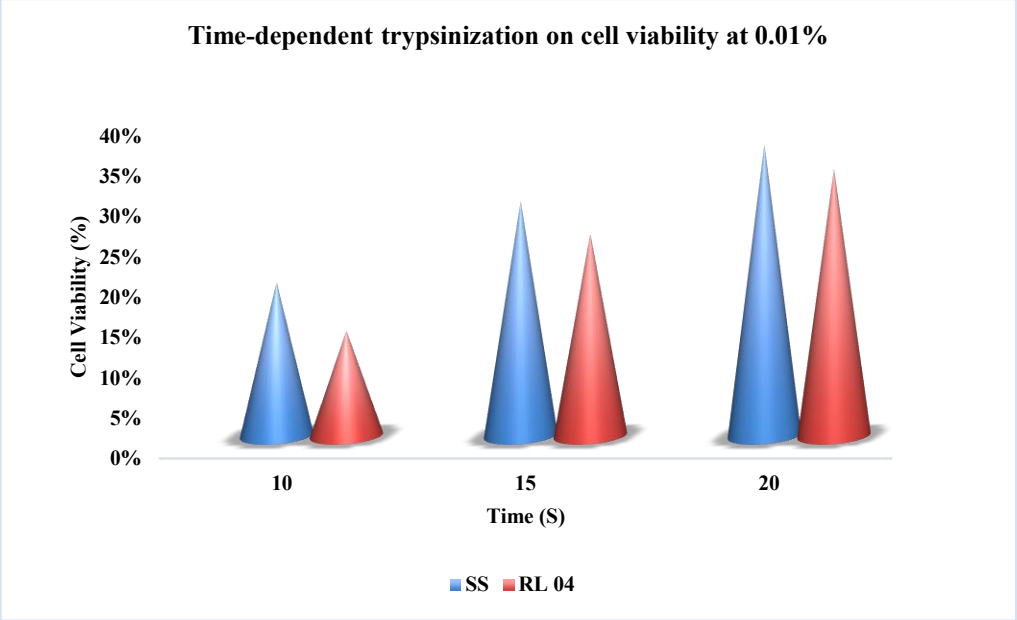

Fig. 1.11Time-dependent trypsinization on cell viability at 0.01%

When cell viability in the presence of 0.1% concentration of trypsin sing agents was monitored (Plate 6), again RL-IV was found to reduce cell viability respectively, by 10%, 20% and 18% at all three time points as compared to 5%, 10% and 3% reduction by RL-IV as shown in Fig. 4.18.

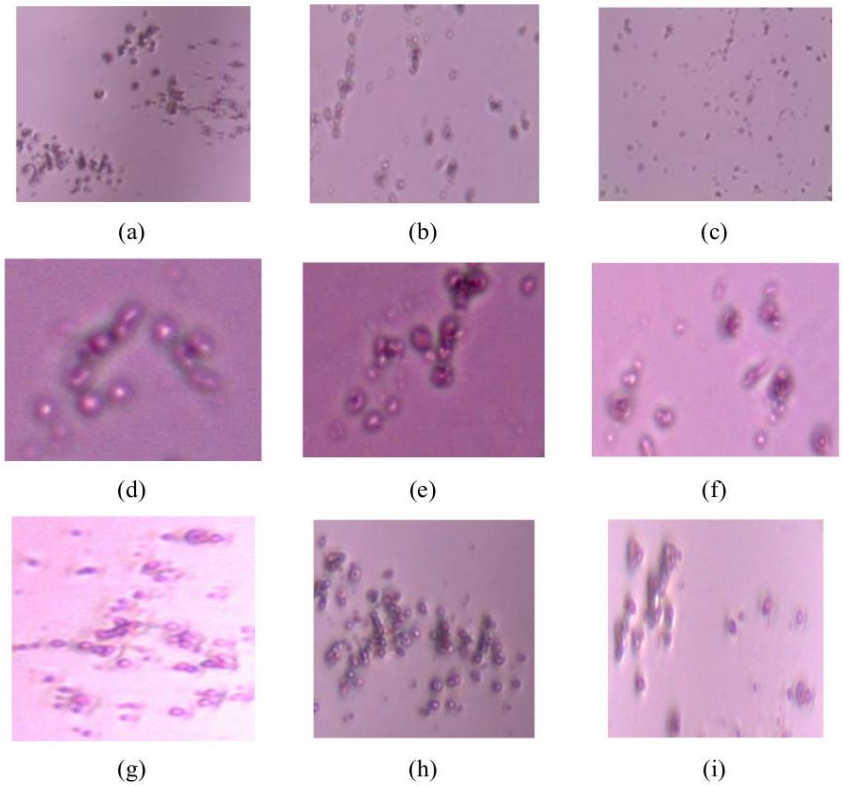

Plate 6: (a) SS for 10 seconds, (b) SS for 15 seconds, (c) SS for 20 seconds, (d) RL-IV for 10 seconds, (e) RL-IV for 15 seconds, (f) RL-IV for 20 seconds, (g) RL-IV for 10 seconds, (h) RL-IV for 15 seconds, and (i) RL-IV for 20 seconds (40X).

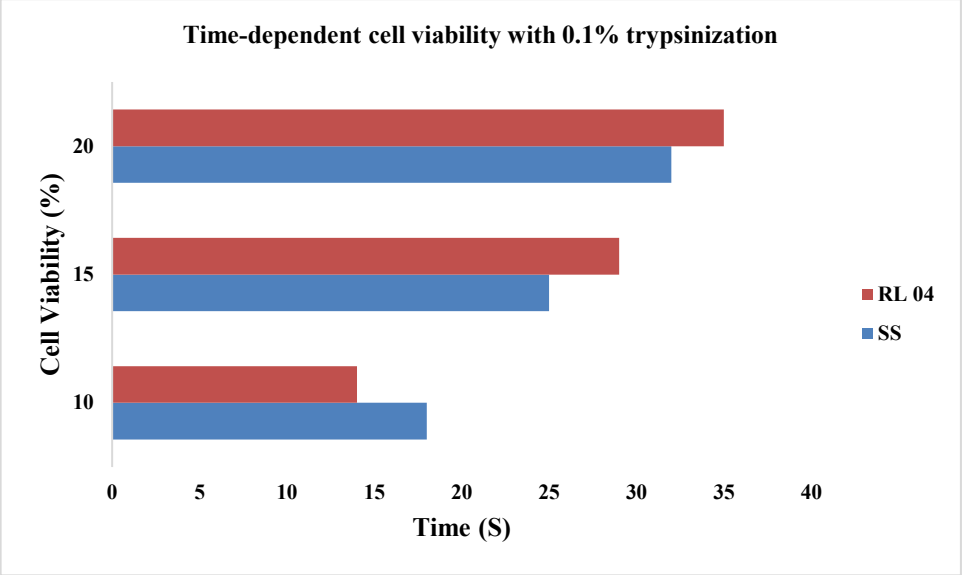

Fig. 1.12Time-dependent cell viability with 0.1% trypsinization

Plate 7 shows cell viability at 1% concentration of trypsinization agents. The trend of reduction in cell viability (Fig. 1.12) of RL-IV was 10%, 14% and 14% respectively at 10, 15 and 20 sec. RL-IV, on the other hand reduced cell viability by only 7%, 2% and 2% respectively at three time points.

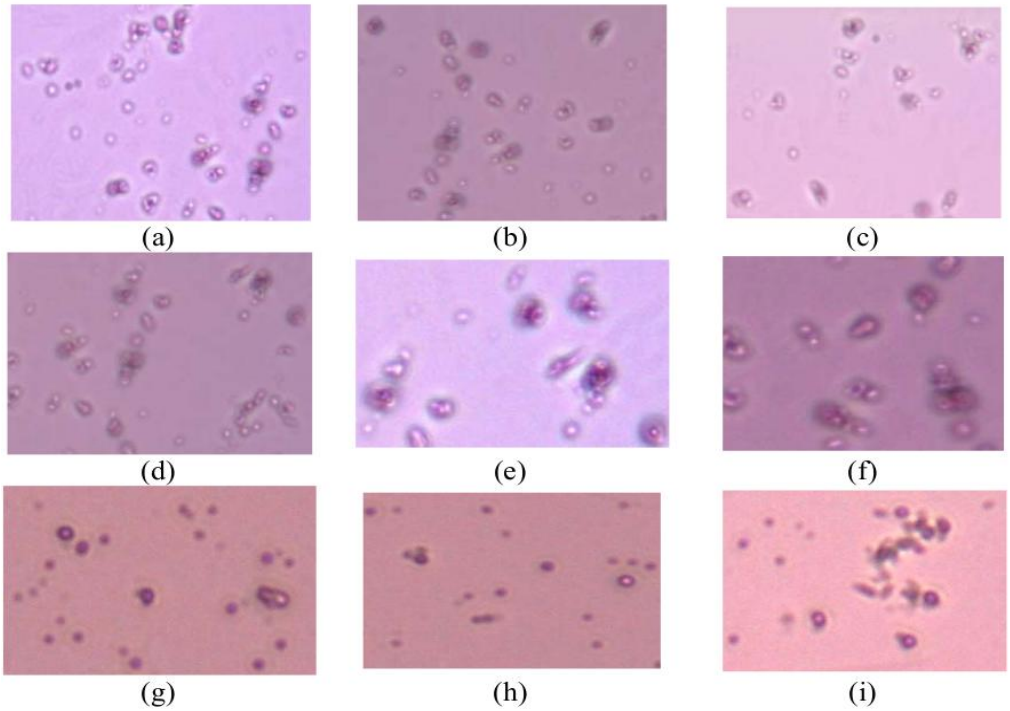

Plate 7: Microphotograph of (a) SS for 10sec, (b) SS for 15sec, (c) SS for 20sec, (d) RL-IV for 10sec, (e) RL-IV for 15sec, (f) RL-IV for 20sec, (g) RL-IV for 10sec, (h) RL-IV for 15sec, and (i) RL-IV for 20sec (40X)

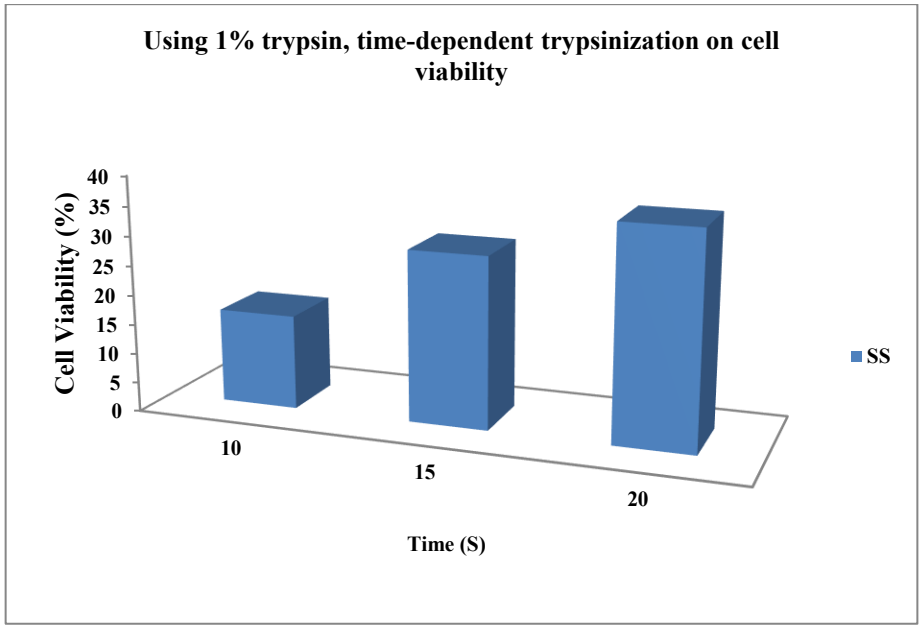

Fig. 1.13Using 1% trypsin, time-dependent trypsinization on cell viability

CONCLUSION

In conclusion, fish visceral hydrolysate of Labeorohita has a wide range of applications in different fields, including aquaculture, food industry, animal feed, and even as a source of fish oil. The hydrolysate is a valuable source of essential amino acids, peptides, and other nutrients that are crucial for the growth and development of fish and shrimp. Its use in aquaculture has been shown to improve the feed conversion ratio, weight gain, and survival rate of fish and shrimp, and can also help to improve water quality and control disease[21].

Furthermore, the potential use of fish visceral hydrolysate of Labeorohita as a source of fish oil can provide a sustainable and low-cost alternative to traditional fish oil sources, while also reducing waste and increasing the value of fish processing. Its applications in different industries have the potential to improve efficiency, promote sustainability, and provide health benefits for both humans and animals.

Overall, the use of fish visceral hydrolysate of Labeorohita can help to address various challenges in different fields, from improving aquaculture operations to providing nutritional supplements and improving animal feed. Further research and development in this area can help to unlock its full potential and promote sustainable and efficient use of fish processing waste[22].

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