Supporting Information

Quantifying the co-solvents effects on trypsin from the digestive system of Carp *Catla Catla* by biophysical techniques and molecular dynamics simulation

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Purification and Characterization of trypsin from Indian Major Carp, *Catla catla*

1. Materials and methods

1.1. Materials

Trypsin was extracted and purified by standard procedure [1] (detailed procedure of extraction and purification is described in the supporting document of this paper). TMAO, betaine, proline, GdnHCl and Tris (hydroxymethyl) aminomethane from Sigma Chemical Co. and urea from Acros Organics were purchased. All chemicals are of analytical grade and used without further purification. Distilled deionized water with resistivity of 18.3 MΩ was used in the preparation of Tris HCl buffer of 1.5 mM at pH 7.5. The concentration of protein was maintained at 3 mg/mL for all measurements.

1.1.1. Purification of trypsin

1.1.2. Maintenance of *fish*

Indian Major Carp, *Catla catla* (catla) was collected from local fish market, INA, Delhi and acclimatized at outdoor conditions for 15 days. Fish was fed with artificial diet containing 40% protein. Artificial diet was prepared by using dried fish powder, wheat flour, cod liver oil and vitamin and mineral premixes. [2] Fishes were kept in fasting condition for 48 h before sampling.

1.1.3. Sampling of tissue

Fish was anesthetized with MS 222 (Tricaine methanesulfonate) before sacrifice. Individual fish was dissected; digestive system and associated glands (hepatopancreas) were removed

from the body, cleaned, weighed and immediately frozen at -20 ^{0}C till further use. Protein concentration was measured by the method of Bradford. [3] Bovine serum albumin was used as a standard.

1.2. Methods

1.2.1. Trypsin activity

Trypsin activity was measured with N-α-benzoyl-DL-arginine-p-nitroanalidine (BAPNA). 750 µl of BAPNA (1 mM in 50 mM Tris-HCl, pH 8.2, 20 mM $CaCl_2$) was incubated with 10 µl of enzyme extract at 37 ^{0}C and change of absorbance was recorded under kinetic mode for 3 min at 410 nm. [4] Trypsin activity was expressed as change in absorbance/min/mg protein of the enzyme used in the assay. Activity units were calculated by the following equation:

$$\text{Activity Units} = \frac{(\text{Abs}_{410}/\text{min}) \times 1000 \times \text{ml of reaction mixture}}{\text{Extinction coefficient of Chromogen} \times \text{mg protein in reaction mixture}}$$

The molar extinction coefficient of paranitroanalidine liberated from chromogens of BAPNA is 8800. Purified trypsin of catla was incubated with various osmolytes such as TMAO, proline, betaine and denaturants such as urea and GdnHCl with concentrations ranging from 1 to 3 M. After incubating for 1 h in respective solutions then the percent relative activity of trypsin was determined.

1.2.2. Preparation of crude *extract*

Digestive system and hepatopancreas collected from more than one fish of same species were pooled; total weight of tissue was recorded and homogenized in sample buffer (10 mM Tris-HCl and 10 mM $CaCl_2$; pH 8.0) in the ratio of 1:3. Homogenized sample was passed through pretreated cheese cloth for the separation of excess fats. The cheese cloth was kept in 1% EDTA solution for 12 h. The suspension was then centrifuged for 30 min at 13000 rpm at 4 ^{0}C. The floating fat phase was removed and the solution was filtered through Buchner funnel (Borosilicate grade-2) under vacuum (ROCKER-300, TARSONS, Mumbai, India), volume of the solution was noted and this sample is called crude extract.

1.2.3. Purification of crude sample

Crude extract is subjected to ammonium sulfate fractionation.[5] Saturated ammonium sulfate (30%) was slowly added to the crude extracts with constant stirring for 2 h after final addition of ammonium sulfate. Then centrifuged at 13000 rpm at 4 ^{0}C for 30 min and the supernatant was brought to 50% saturation by further addition of ammonium sulfate. Then the sample was centrifuged at 13000 rpm at 4 ^{0}C for 30 min and the precipitate was collected; resuspended in the minimal volume of sample buffer. The sample was dialyzed (Sigma, D

0530, 12.4 kDa) overnight against the same buffer and was filtered through 0.45 µm Polyethersulfone membrane (25 mm diameter, Whatman) syringe filter. Trypsin activity and protein concentration of filtrate were assayed. Filtrate was applied slowly (0.25 ml/min) to a DEAE-Cellulose column (0.5 × 5.5 cm, Bio-Rad, USA), which was previously equilibrated with same sample buffer. The column was washed with the equilibration buffer or sample buffer until the effluent had no detectable absorption at 280 nm. The whole process was carried out at 4 ^{0}C.

Trypsin-like enzymes were then eluted from the column using a step gradient of NaCl with different concentrations ranging from 100 to 500 mM NaCl in the starting buffer. Finally, the column was washed with 1 M NaCl in the starting buffer. The flow rate was adjusted to 24 ml/h and 4.5 ml fractions were collected. The eluents were monitored at 280 nm for protein and at 410 nm for trypsin activity. Highest activity fractions were pooled as PF1 and further processed with affinity chromatography.

The pooled fraction was adjusted to 0.2 M KCl by the addition of solid salt and applied to a Benzamidine Sepharose 4 Fast flow (high sub) Amersham Biosciences, Sweden, column (1.6 × 2.5 cm, 5 ml) which was equilibrated previously with 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ and 200 mM KCl. The column was then washed with the same buffer. Sample was applied at 0.25 ml/min and the column was washed at a flow rate of 20 ml/h and 2.5 ml fractions were collected. Trypsin was eluted from the column by 0.1 M acetic acid. The fractions eluted from the column were immediately adjusted to pH 8.0 by adding 700 µl of 1 M Tris-HCl buffer (pH 9.0).

1.2.4. Inhibition study

The effect of inhibitors on enzyme activity was examined by treating the enzymes with different specific inhibitors.[6] Soyabean trypsin inhibitor, SBTI (250 µm SBTI, Sigma, St. Louis, USA) in 1 mL distilled water and N α-p-tosyl-L-lysine chloromethyl ketone, TLCK (10 mM TLCK, Sigma, St. Louis, USA) in 1 mL of 1 mM HCl were used as specific inhibitors for serine protease inhibitor and trypsin inhibitor, respectively. These inhibitors were pre-incubated at 1:1 ratio for 1 h at room temperature. The pre-inhibited samples were subjected to trypsin activity with BAPNA at 25 ^{0}C (pH 8.2) and the percentage of inhibition was recorded.

1.2.5. Inhibition study by substrate SDS-PAGE

Solutions of inhibitors like SBTI (250 µM) and TLCK (10 mM) were prepared and 10 µl of each inhibitor solution was incubated with 10 µl enzyme preparations containing 5 mUnits activity at 25 ^{0}C for 1 h prior to loading onto the wells. Then they were subjected to substrate

SDS-PAGE. Resulting bands were compared with activity bands of the enzyme preparation without inhibition. The gels were documented in calibrated densitometer (GS-800, Bio-Rad, CA 94547, USA) with help of Quantity one - 4.5.1 software.

1.2.6. SDS-PAGE and substrate SDS-PAGE

Separation of proteins in the enzyme extracts was done by 12% SDS-PAGE according to Laemmli.[7] Enzyme extract (20 µg protein/sample) was loaded onto each well and electrophoresis was performed (30 mA) on a vertical dual mini gel electrophoresis device (Hoefer SE-260, Amersham Pharmacia, Uppsala, Sweden) at a controlled temperature of 4 ^{0}C. The protease composition was studied after separation of proteins by substrate SDS-PAGE,[8] samples containing 5 mUnits of activity was loaded onto each well. After electrophoresis, the gel was immersed in a solution of 3% casein and incubated for 60 min at 25 ºC. The gel was then washed, stained with 0.1% Coomassie brilliant blue (CBB, Mumbai, India) in methanol: acetic acid: water (40:10:40) for 2 h. Destaining was done with the same solution without CBB for 1 h. Clear bands with blue background were identified as protease activity bands. The gels were documented in calibrated densitometer (GS-800, Bio-Rad, CA 94547, USA) with help of Quantity one - 4.5.1 software.

2. Results

2.1. Purification of trypsin

The overall purification of trypsin was 107.4-fold, with the recovery of 24.61% (Table 1). A purification fold of 5.1 was obtained with the yield of 50.81%, when ammonium sulphate at 30-50% saturation was used followed by dialysis, suggesting the removal of some contaminating proteins and salts from the crude extract. As the dialysate sample was applied on to DEAE-Cellulose column, single activity peak (Fig. 1) was obtained in 150 mM NaCl fractions (tube nos. 49-56). Highest activity fractions were pooled as PF1; DEAE-Cellulose purification results into the yield of 48.05% with purification fold of 91. Purified fraction (PF1) of ion exchange chromatography, was subjected to affinity chromatography using PABA-Sepharose 4B; single activity peak was observed (Fig. 2) at fraction number 30. This suggests efficacy and specificity in binding of trypsin by this column. The content of tube which contained high activity was pooled as PF2. The obtained Trypsin was purified by 107.4-fold with recovery being 24.61%. However, unlike most purification procedures, one should take extra care in dealing with trypsin-like proteases, which are extremely susceptible to autolytic degradation. It has been difficult to detect zymogens in pancreatic extracts of fish and enzymes appear to be fully active when first isolated.[9-12] This is used to separate active

biomolecules from denatured or functionally different forms, to isolate pure substances present at low concentration in large volumes and also to remove specific contaminants. Because of the high specificity of this column (affinity chromatographic matrix) to bind the trypsin, other enzymes or proteins in PF1 fraction could not bind to the column and were easily washed out. Only trypsin was eluted during elution process. This led to the high purity of trypsin after this affinity chromatography step.

2.2. Effect of inhibitors

Protease inhibitors are very important tools used to characterize enzyme active sites and to understand enzymatic mechanisms. The effects of protease inhibitor (SBTI) and specific trypsin inhibitor (TLCK) on the activity of trypsin were determined. Trypsin activity was inhibited (99.6%) by 1.0 g/l SBTI and completely inhibited (100%) by 10 mM TLCK. The enzyme was effectively inhibited by SBTI and TLCK. The inhibition study indicated that purified enzyme was a serine protease based on inhibition by PMSF and authentic trypsin based on its catalytic specificity for BAPNA and susceptibility to inhibition by TLCK and SBTI.

The inhibition results indicated that purified enzyme was a serine protease based on inhibition by PMSF and authentic trypsin based on its catalytic specificity for BAPNA and susceptibility to inhibition by TLCK and SBTI. A trypsin-like enzyme from tambaqui pyloric caeca was inhibited by trypsin inhibitors, such as PMSF, benzamidine and TLCK. [13] Two kinds of trypsins from the spleen of yellow fin tuna were inhibited by SBTI and TLCK. [14] Trypsin was markedly inhibited by trypsin inhibitors such as soybean trypsin inhibitor (93.75% inhibition) and TLCK (90.50% inhibition). No inhibition was observed when TPCK, a specific inhibitor for chymotrypsin, was used. [15]

2.3. SDS-PAGE

Generally, trypsins have been reported to have molecular masses between 20 and 30 kDa. [16] The SDS-PAGE of purified trypsin is shown in Fig. 3. A commercial preparation of bovine trypsin was included for comparison with the standard molecular weight marker of low range (Bio-Rad, USA). In the crude extract, there are total eight bands, with three high molecular weights of 117.09, 103.3 and 101.96 kDa. Five distinct bands are appeared with molecular weights of 72.96, 40.19, 26.82, 23.54 and 19.94 kDa. Protein bands were purified and reduced to four in ion-exchange chromatography, PF1. In PF1, one prominent low molecular weight band (19.91 kDa) was observed along with three high molecular weight bands of 117.09, 103.3 and 102 kDa. SDS-PAGE of affinity fraction PF2 purified to homogenous single band with molecular weight of 19.72 kDa. These results are similar to trypsins from

feline (21 kDa), [17] tongol tuna (21 kDa) and yellow tuna (21 kDa). [13] However, the molecular masses are lower than those of trypsins from common carp (28 kDa and 28.5 kDa) [18], rainbow trout (25.7 kDa), [19] Atlantic bonito (29 kDa) [20] and walleye pollock (24 kDa) [21]. The differences of molecular mass in trypsin may be due to genetic variation among species. However, the possibility that these differences are caused by autolytic degradation should not be excluded.

2.4. Substrate SDS-PAGE and inhibition in zymogram

Zymogram of crude and uninhibited sample shows (Fig.4) four activity bands of molecular weights of 44.50, 31.6, 20.86 and 18.61 kDa. Among these four activity bands TLCK inhibited two activity bands of molecular weight 20.86 and 18.61 kDa, whereas SBTI inhibited only one activity band of molecular weight 32.6 kDa. Single activity band was observed in the purified sample (affinity fraction, PF2) with molecular weight of 19.1 kDa. This activity band was completely inhibited by the TLCK treated sample and SBTI treated sample showed (95%) inhibition with single activity band of molecular weight 19.6 kDa. These results for molecular weight and effects of inhibitors, confirmed that the purified enzyme was serine protease. TLCK is well known as a trypsin specific inhibitor. TLCK inactivates only trypsin-like enzymes by forming a covalent bond with histidine at the catalytic portion of molecule and then blocking the substrate-binding portion at the active center. [22] Two kinds of trypsins from the hepatopancreas of carp were inhibited by some trypsin inhibitors, such as soybean trypsin inhibitor, aprotinin, benzamidine and TLCK. [18] Trypsin-like enzyme from tambaqui pyloric caeca was inhibited by some trypsin inhibitors, such as PMSF, benzamidine and TLCK. [13]

Table 1S. Transition temperature (T_m), enthalpy change (ΔH), and heat capacity change (ΔC_p) determined by Fluorescence spectroscopy and calculated Gibbs free energy changes in unfolding state (ΔG_u) at 25 °C for the trypsin in different solvent media.

sample[a]	T_m (^{0}C)	ΔH (kJ.mol^{-1})	ΔG_u (kJ.mol^{-1})	ΔC_p (kJ.mol^{-1}.K^{-1})
Buffer	58.5	129	20.56	0.389
1 M TMAO	59.1	131	20.96	0.396
2 M TMAO	60.0	132	21.45	0.397
3 M TMAO	61.0	134	21.98	0.401
1 M Proline	60.0	133	21.64	0.400
2 M Proline	60.8	135	22.19	0.405
3 M Proline	62.0	136	22.52	0.408
1 M Betaine	60.5	137	22.49	0.409
2 M Betaine	61.7	139	23.07	0.413
3 M Betaine	63.3	140	23.67	0.418
1 M urea	58.0	126	20.01	0.381
2 M urea	57.5	124	19.52	0.376
3 M urea	56.6	122	19.02	0.371
1 M GdnHCl	57.5	125	19.71	0.378
2 M GdnHCl	57.0	122	19.11	0.370
3 M GdnHCl	55.7	119	18.34	0.363

[a]Each Value is the average over three measurements. The error in Tm doesnot exceeds 0.1^{o}C. The estimated relative uncertainties in (ΔH), (ΔC_p) and (ΔG_u) are around 2% of the reported values.

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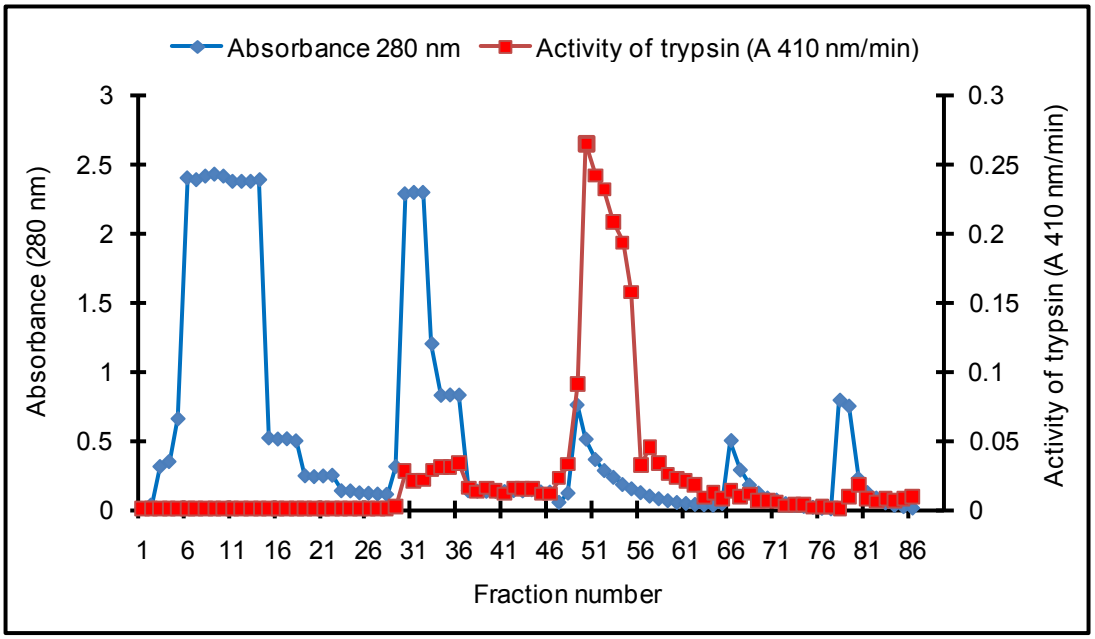
Figure Captions

Fig. 1. Elution profile of trypsin of *Catla catla* on DEAE-Cellulose column. Elution was performed with step gradient of NaCl (100 - 500 mM) in the equilibration buffer (10 mM Tris-HCl, pH 8.0 containing 10 mM $CaCl_2$). Protein content was expressed in absorbance at 280 nm and activity in terms of absorbance at 410 nm/min.

Fig. 2. Affinity chromatography (PABA-Sepharose) of trypsin of *Catla catla.* Elution was carried out with 0.1 M CH_3COOH. Protein content was expressed in absorbance at 280 nm and activity in terms of absorbance at 410 nm/min.

Fig. 3. SDS-PAGE of enzyme sample of digestive tissue of Catla catla at various stages of purification. Sample was diluted (1:1) with sample buffer. MWM comprising Phosphorylase b (97,400); bovine albumin (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); trypsin inhibitor (21,500); and lysozyme (14,400). After electrophoresis the gel was directly stained with Coomassie brilliant blue R-250 for 2 h and destained. M -Bio Rad Marker Low range, CE - Crude Extract, PF1 (IEC) - Purified Fraction (Ion exchange chromatography), PF2 (AF) - Purified Fraction (Affinity chromatography) and ST - Standard trypsin (bovine).

Fig. 4. Substrate SDS-PAGE of crude extract and purified sample of Catla catla. Sample was diluted (1:1) with sample buffer. MWM comprising Phosphorylase b (97,400); bovine albumin (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); trypsin inhibitor (21,500); and lysozyme (14,400). Electrophoresis was done at 4 °C. Electrophoresed gel was incubated with 3% casein for 1 h, then washed and directly stained with Coomassie brilliant blue R-250 for 2 h and destained. M - Bio Rad Marker Low range, CE (UI) - Crude Extract Uninhibited (Protease activity), CE (TLCK) - Crude Extract (N α-p-tosyl-l-lysine chloromethyl ketone) Inhibition, CE (SBTI) - Crude Extract (Soybean trypsin inhibitor) Inhibition, PF (UI) - Purified Fraction Uninhibited (Protease activity), PF (TLCK) - Purified Fraction (Nα-p-tosyl-L-lysine chloromethyl ketone) Inhibition, PF (SBTI) - Purified Fraction (Soybean trypsin inhibitor) Inhibition.


Absorbance 280 nm
Activity of trypsin (A 410 nm/min)
Absorbance (280 nm)
Activity of trypsin (A 410 nm/min)
Fraction number
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0.5
1
1.5
2
2.5
3
0.05
0.1
0.15
0.2
0.25
0.3
1
6
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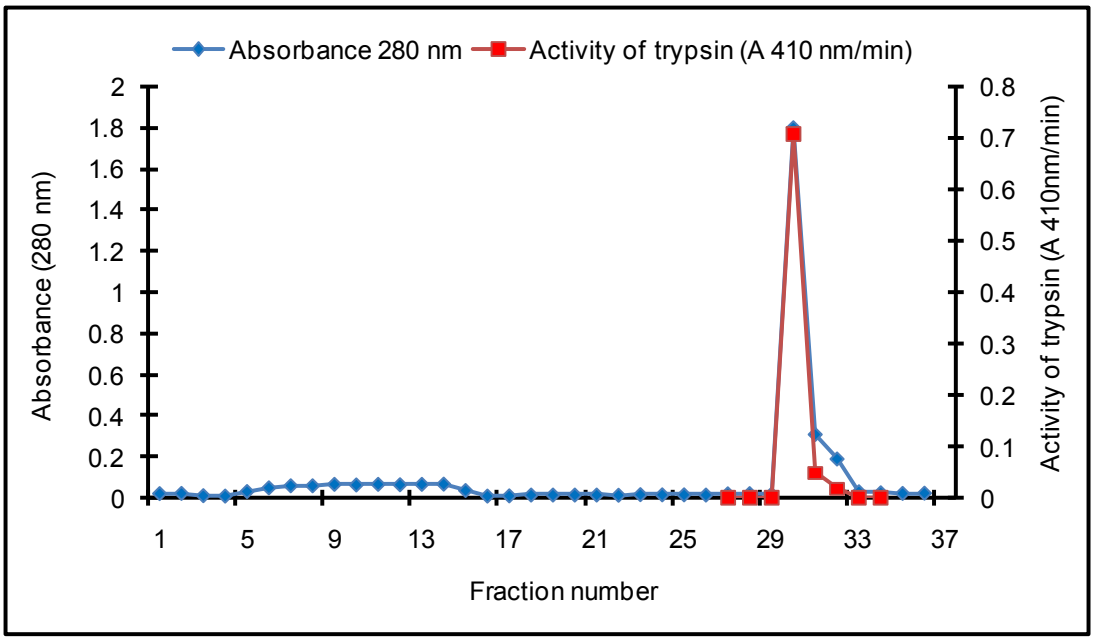
71
76
81
86

Fig. 1.


Absorbance 280 nm
Activity of trypsin (A 410 nm/min)
Absorbance (280 nm)
Activity of trypsin (A 410nm/min)
Fraction number
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.8
0.7
0.6
0.5
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33
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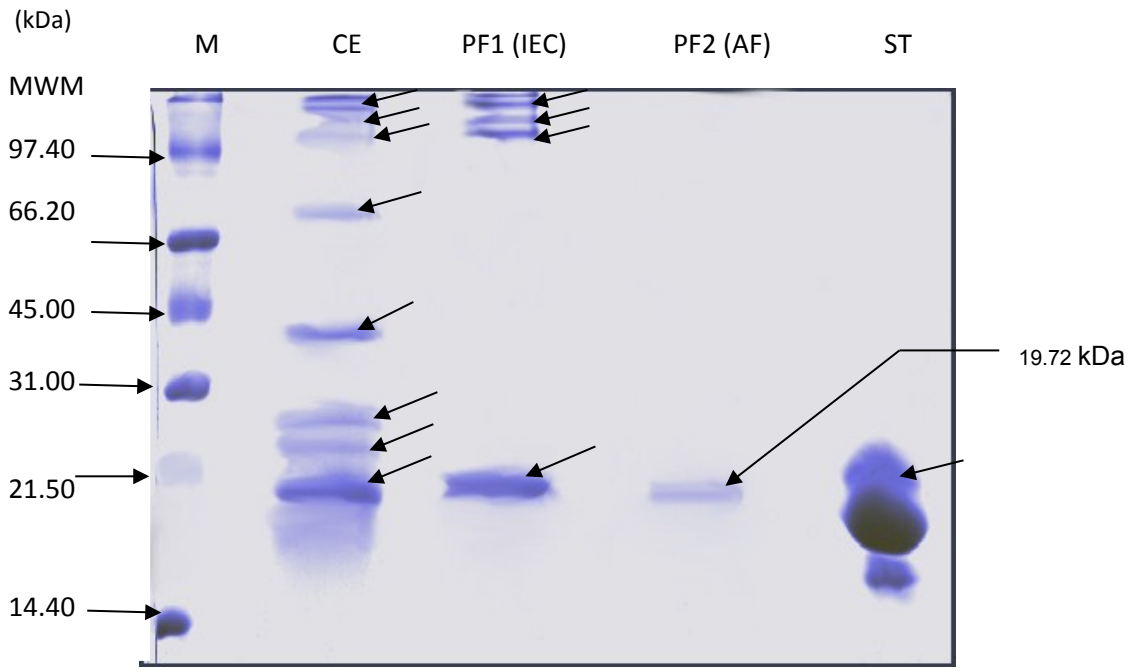
Fig. 2.


(kDa)
M
CE
PF1 (IEC)
PF2 (AF)
ST
MWM
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66.20
45.00
31.00
21.50
14.40
19.72 kDa

Fig. 3.

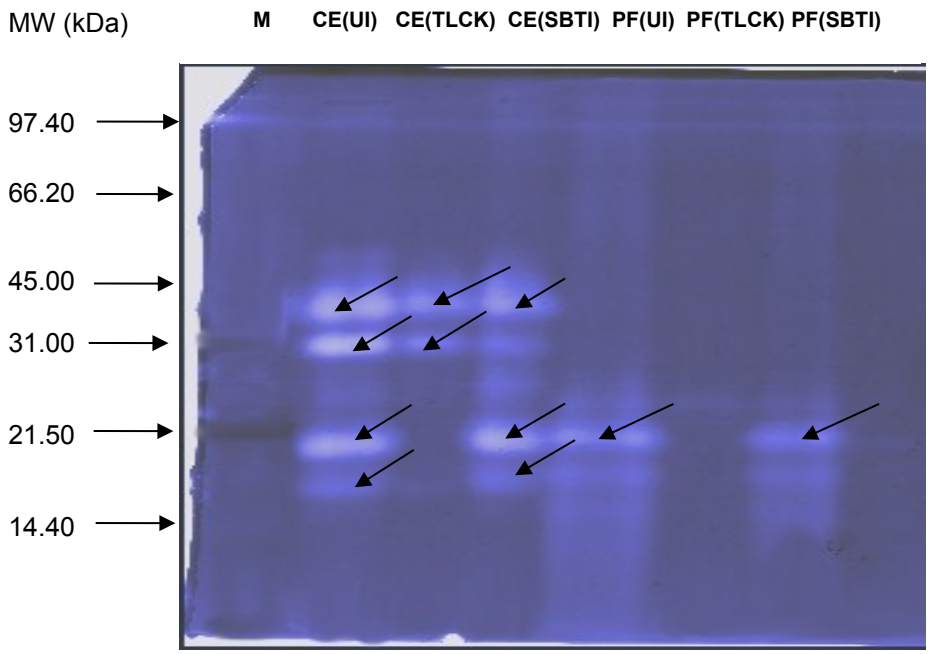
MW (kDa)
M
CE(UI)
CE(TLCK)
CE(SBTI)
PF(UI)
PF(TLCK)
PF(SBTI)
97.40
66.20
45.00
31.00
21.50
14.40

Fig. 4.