

Carboxypeptidase-B from *Bubalus bubalis* pancreas: Purification, properties and MALDI-TOF monitored activation of proinsulin

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Abstract: Carboxypeptidase-B (E.C 3.4.17.2) catalyzes the hydrolysis of peptides and esters at C-terminus of arginine and lysine residues. Our study describes the large scale purification, N-terminal sequence analysis and physiochemical properties of pancreatic enzyme from river buffalo (*Bubalus bubalis*). The enzyme was purified up to 71 folds by anion-exchange chromatography with 21% final recovery. Purified enzyme displayed two bands on SDS-PAGE with molecular weights of 9 kDa and 26 kDa respectively, the N-terminal sequence of later was EFLDKLDFYV. The enzyme has shown optimum activity at pH 9.0 and 40°C. The K_M , K_{cat} and K_{cat}/K_M values of purified carboxypeptidase-B with Hippuryl-L-Arg are 30 μ M, 72sec⁻¹ and 2.4x10⁵ M⁻¹ sec⁻¹ respectively. A computer based model for the structure of enzyme was proposed by chromatographic studies of component fragments and N-terminal sequence. The enzyme purified in the present study was free of carboxypeptidase A and endoprotease contamination. It was efficiently used in the processing of recombinant buffalo proinsulin, in combination with trypsin. Activation of proinsulin was monitored by MALDI-TOF analysis of peptides before and after the action of enzymes.

Keywords: CpB, river buffalo, N-terminal sequence, proinsulin processing, MS analysis.

INTRODUCTION

Carboxypeptidase-B (CpB) is a metalloenzyme produced in mammalian pancreas as a monomeric zymogen which requires a zinc atom per molecule for its functioning. Naturally, the active enzyme helps in the further degradation of products of tryptic digestion in the small intestine of mammals (Aviles *et al.*, 1993). The pro-peptide which is lost during the activation of procarboxypeptidase-B is considerably longer than that of other proteolytic enzymes which are produced as zymogens (Burgos *et al.*, 1991; Yamamoto *et al.*, 1992). Studies on the activation of procarboxypeptidase-B have shown that the action of trypsin on zymogen is very quick at 37°C and pH 7.5 which gives complete activation in very short time if the trypsin and zymogen ratio is adjusted at 1:40 respectively (Burgos *et al.*, 1991; Kylanpaa-Back *et al.*, 2002).

Carboxypeptidase-B has been purified by chromatographic techniques such as zinc chelate chromatography (Hortin and Gibson, 1989), metal chelation affinity chromatography (Marquez-Mendez, 1992) and CM Sepharose CL-6B column based chromatography (Honda *et al.*, 2000). The molecular weight of active CpB has been reported between 31kDa to 36.4kDa (Ferrel *et al.*, 1975; Marinkovic *et al.*, 1977; Bradley *et al.*, 1996). CpB has clinical and pharmaceutical importance, the activation peptide of enzyme has been used as biomarker of acute pancreatitis (Mayer *et al.*, 2002; Saez *et al.*, 2004). The enzyme is also used for the removal of Arg³¹/Arg³² in the process of proinsulin activation (Son *et al.*, 2009).

B. bubalis is an animal of great importance in livestock and dairy which also contributes as predominant slaughter house animal in South Asia. In Pakistan, the buffalo population is 26.3 million consisting of Nili, Ravi, Nili-Ravi and Kundi breeds which contribute about 68% of milk and 32% of total meat production in the country (Bilal *et al.*, 2006; Khan and Iqbal, 2009). Our research project aimed to explore the characteristics of *B. bubalis* enzymes and proteins has already reported the purification and properties of heart aspartate aminotransferase (Nadeem *et al.*, 2011), lactate dehydrogenase (Nadeem *et al.*, 2011) and mitochondrial malate dehydrogenase (Nadeem *et al.*, 2012). The nucleotide sequences of complete cDNA molecules encoding these enzymes have also been reported. Present study describes the characteristics of pancreatic CpB which can be useful in cataloguing *B. bubalis* for its enzymes. Our study also describes the use of purified CpB in the processing of recombinant river buffalo proinsulin which was monitored by MALDI-TOF analysis at every important step to elaborate the particular role of CpB in the process.

MATERIALS AND METHODS

Hippuryl-L-Arg, Diethylaminoethyl Sephadex (DEAE-Sephadex) and chromatography related reagents were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, Mo. USA) and Calzyme (Calzyme Laboratories, Inc., San Luis Obispo, CA, USA). Fresh pancreatic tissue was obtained from the main slaughter house in Lahore, Pakistan.

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Purification of Enzyme

Fresh pancreatic tissue (1000g) was minced, kept at room temperature for 24 hrs for autolysis and blended in equal volume of ice-chilled acetone in a waring blender at high speed for one min. The blended tissue sample was filtered through Whatmann filter paper No-113 by suction filtration, the residue was re-extracted and blended in 2 volumes of ice-cold acetone. The process of blending and re-extraction was repeated once with 3 volumes of acetone, then with 2 volumes of acetone-ether (1:1, V/V) finally with 3 volumes of ether. The extract of acetone powder was prepared by mixing 100 g of powder in 1500 mL of ice chilled 10 mM Tris-HCl buffer pH 7.5 (buffer-A) in the ice box for 40 min. The suspension was centrifuged at 9000x *g* for 15 min at 4°C and residue was discarded and light-yellow clear supernatant was used for the purification of enzyme.

The supernatant was chilled to 0°C and solid ammonium sulfate (NH₄)₂SO₄ was added to the preceding solution to give 30% saturation. The precipitate was removed by centrifugation at 9000x *g* and 4°C for 20 min and supernatant was brought from 30% to 65% ammonium sulfate saturation. The precipitate was separated by centrifugation at 12000x *g*, 4°C for 20min and supernatant discarded. Final precipitate was dissolved in and dialyzed against buffer-A. DEAE-Sephadex column was equilibrated with dialyzing buffer and sample was applied to the column at a flow rate of 3 mL per min. The column with bound proteins was washed with buffer and eluted with 0.5 M NaCl linear gradient in buffer-A. Fractions were collected and those with high enzyme activity were combined and analyzed on SDS-PAGE.

Enzyme assay

Enzyme activity was measured with the method based on the spectrophotometric property of acids, esters and amides that have approximately same absorbance maxima. However, the extinction coefficient of acids is higher than that of their related esters and amides. The activity was measured by an increase in absorbance at 254 nm resulting from the hydrolysis of Hippuryl-L-Arg to L-Arg and Hippuric acid. The assay reaction mixture containing 600μM Hippuryl-L-Arg in 50mM Tris-HCl buffer pH 8.0 was added to the experimental and control cells. Both cells, containing the reaction mixture were placed in a spectrophotometer (SHIMADZU BioSpec-1601) at 35°C for 5 min to observe any change in absorbance. The experimental cell was added with 10μl of carboxypeptidase-B dilution, change in absorbance at 254 nm was monitored for 4 min and the micromoles of Hippuryl-L-Arg consumed per min were calculated. Total protein content of the solution was calculated by Bradford method (Bradford, 1976).

Molecular weight and structural analysis

Purified enzyme displayed two protein bands on SDS-PAGE. The high molecular weight fragment was excised

from the PAGE and analyzed for N-terminal sequence (by Alta Bioscience University of Birmingham United Kingdom). The possible interaction between larger and smaller fragment was determined by exposure of active enzyme sample to the denaturing and non-denaturing conditions. The purified enzyme sample was subjected to gel-filtration chromatography on Superdex G-200 column on FPLC using 50 mM Tris-HCl buffer pH 8.0 containing 140 mM NaCl as the mobile phase at a flow rate of 0.5 mL per min (non-denaturing conditions). In parallel, it was also subjected to hydrophobic interaction chromatography on C-18 hydrophobic column on HPLC. The mobile phase included 0.1% TFA (trifluoroacetic acid). Prelude to the application of sample the HPLC column was washed with 80% acetonitrile gradient prepared in 0.1% TFA. The sample containing 4.0 mg of protein was loaded onto the column and the bound protein was eluted between 35% to 80% gradient of acetonitrile. The protein profile was monitored at 220 nm and the chromatograms were saved. The purified protein peaks eluted at different retention times were collected and analyzed on SDS-PAGE. A computer based model of procarboxypeptidase B was suggested on the basis of these studies using the amino acid sequence of enzyme from *Bos taurus*.

Kinetic properties of enzyme

The effect of pH and temperature was determined by using buffer solutions of various pH (6-11) for the preparation of reaction mixture and the incubation of standard assay solution at different temperatures respectively. For the determination of the effect of metal ions and L-Arg, the assay mixture containing 50 μM Ca, Zn, Mg, Ni, Co, Mn ions and L-Arg were used separately. A control with standard reaction was used for each metal ion. The effect of EDTA was determined on the enzyme activity by incubation of dilute enzyme sample at 4°C in the presence of 50 mM EDTA for 5, 10, 15, 20, 25 and 30 min and the activity was measured. K_M, V_{max} and K_{cat} values of the purified enzyme were also determined.

In vitro activation of proinsulin

A derivative of buffalo proinsulin containing N-terminal methionine, six histidine and one arginine residues [Met (His)₆ Arg - Proinsulin] was used in the present study. For *in vitro* conversion of proinsulin into insulin, the purified CpB was used in combination of trypsin (Sigma Cat. No. T1426). In the first step, only CpB purified in the present study was incubated with proinsulin derivative at 37°C for 60 min to observe contamination of endoproteases. In the second step, the action of purified CpB and trypsin was observed. The samples were analyzed on MALDI-TOF after 5min and 15min post incubation. Number of peaks and related molecular weights of peptides were determined. The purified samples of proinsulin derivative (100μg) were dissolved in 20 mM Tris-HCl pH 8.0 to make a final concentration of 1.65 μg per μL. In one of the samples, only 0.022 units (1μL) of purified CpB was

added to find out the presence of endoprotease contamination in the purified enzyme and in the other sample 0.5 μg (1 μL) of trypsin was added to make 1:200 ratio of enzyme/substrate (W/W) along with 0.022 U of purified CpB. The samples were incubated at 37°C and 5 μL of each sample was mixed with 12 μL of matrix A (8 mg cinnamic acid in 50 % acetonitrile per 0.3% TFA) to terminate the reaction after 0, 15, 30 and 60 min, 2.0 μL of each sample was spotted in duplicate on MTP 384 polished steel TF target plate and allowed to dry completely. The samples were analyzed by Matrix Assisted Laser Desorption Ionization Time of Light (MALDI-TOF) mass spectrometry using Autoflex III smart beam in LP-Clin Port method using 50 to 60% laser intensity, 293 shots and 2000 Da to 10000 Da molecular weight range.

RESULTS

Purification and N-terminal sequence

The enzyme was purified using anion-exchange chromatography, purified fractions were combined and activity was measured. The purity of final sample was determined on SDS-PAGE which displayed protein bands at 26 kDa and 9 kDa (fig. 1). N-terminal sequence of the 26 kDa fragment excised from SDS-PAGE was found as EFLDKLDFYV. Under non-denaturing conditions on FPLC based gel-filtration column the purified enzyme was eluted as a single peak whereas under denaturing conditions on HPLC based hydrophobic interaction chromatography it was eluted as two separate peaks. The enzyme eluted from Gel-filtration column gave two bands on SDS-PAGE whereas the smaller and larger fragments were observed in separate fractions from hydrophobic interaction column (fig. 2). A computer based model for the zymogen was proposed on the basis of structural studies and N-terminal sequence of 26 kDa fragment (fig. 3).

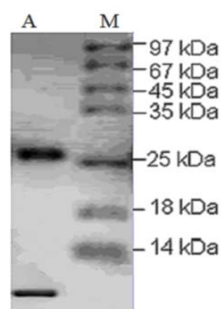


Fig. 1: SDS-PAGE photograph. Lane A-Two bands of purified carboxypeptidase-B from the pancreas of *Bubalus bubalis*, M- Protein marker (14 kDa to 97 kDa).

Kinetics of purified CpB

Carboxypeptidase-B purified in the present study has shown activity at a broad range of pH with an optimum pH of 9.0. Maximum activity was measured at 40°C and it was completely inactivated by incubation at 70°C for 5

min (fig. 4). The activity of purified enzyme was enhanced in the presence of 50 μM of Ca^{2+} and Co^{2+} ions and decreased in the presence of the same concentration of L-arginine. No significant decrease in the activity was measured while the dilute solution of enzyme was incubated in 50 mM EDTA for 30 min at 4°C. K_M and K_{cat} values of enzyme for Hippuryl-L-Arg were 30 μM and 72 sec^{-1} respectively. The enzyme has shown specificity constant (K_{cat}/K_M) of $2.4 \times 10^5 \text{ M}^{-1}\text{Sec}^{-1}$. No activity was observed with Hippuryl-L-Phe (substrate of carboxypeptidase-A).

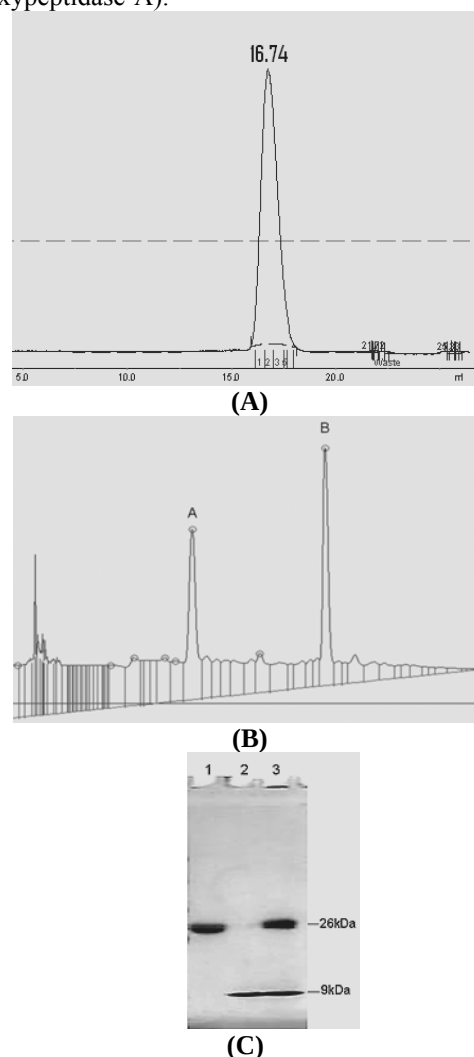


Fig. 2: Elution peaks for chromatography columns under non-denaturing and denaturing conditions using purified enzyme as sample and SDS-PAGE analysis. (A). Single elution peak for gel-filtration chromatography with FPLC. (B). Results of hydrophobic interaction chromatography with C-18 column on HPLC under denaturing conditions. (C). Lane 1 and 2- SDS-PAGE analysis of fractions A and B eluted from HIC-Column by HPLC. Lane 3- SDS-PAGE analysis of single peak eluted from FPLC based gel-filtration chromatography.

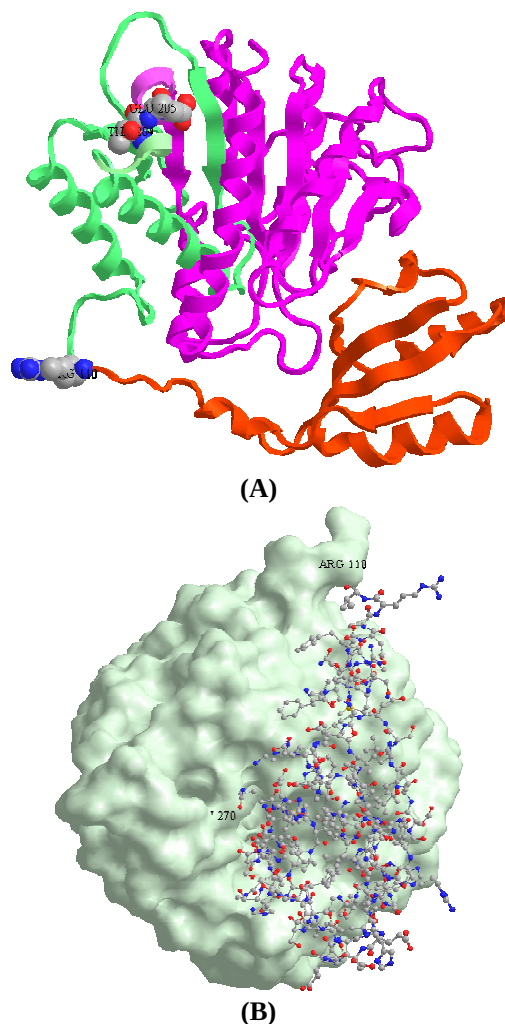
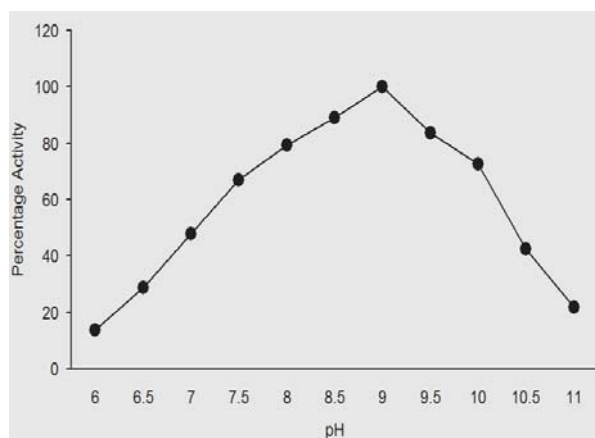


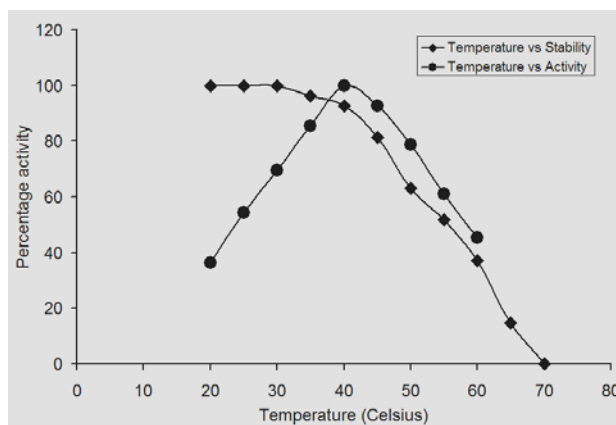
Fig. 3: Purposed model of procarboxy-peptidase-B. **(A)** Three main parts of the zymogen, the activation peptide (red), smaller fragment (green), larger fragment (purple) **(B)**-The smaller and larger components comprising the active enzyme with an active site pocket which remains covered by the activation peptide and makes it inaccessible for the substrate in zymogen.

Proinsulin Processing

Proinsulin is a precursor of insulin which is composed of three amino acid chains, chain A-(21 amino acids), chain B-(30 amino acids) and chain C-(26 amino acids). Chain C connects the A and B chains via two pairs of basic amino acids. Arg³¹, Arg³² and Lys-59, Arg-60. A derivative of river buffalo proinsulin [Met (His)₆ Arg-Proinsulin] was converted to active insulin by using purified CpB in combination with trypsin (Sigma-Aldrich Cat. No. T1426). The action of trypsin gives intermediates which retain one or both arginine residues at position 31 and 32 at the C-terminal of chain B. These two residues are removed by the action of pancreatic CpB. Trypsin also removes N-terminal methionine, His-tag and arginine (fig.



(A)



(B)

Fig. 4: Effects of pH on the activity of purified carboxypeptidase-B. **A.** Optimum pH for the enzyme activity is 9.0. **B.** The effect of temperature on the activity and stability of purified CpB. The optimum temperature for the enzyme activity is 40°C. The enzyme has shown activity at a wide range of temperature and inactivated completely while incubated at 70°C for 5 min.

5). Theoretical molecular weight of recombinant proinsulin used in the present study was 9791.1Da and that of active insulin was 5733.5 Da as calculated by ProtParam. In an initial experiment the purified CpB was incubated with proinsulin derivative at 37°C for 60 min, no change was observed in the ms spectrum indicating the presence of intact proinsulin derivative and not even traces of insulin which proves that the purified CpB was free of endoprotease contamination (fig. 6). The removal of C-terminal Arg31/Agr32 or both was checked out by the incubation of both trypsin and CpB with proinsulin derivative. MALDI-TOF analysis after 5 and 15 min post incubation has shown that the proinsulin (9791.1 Da) was converted into insulin (5733 +/- 2 Da) (fig. 7).

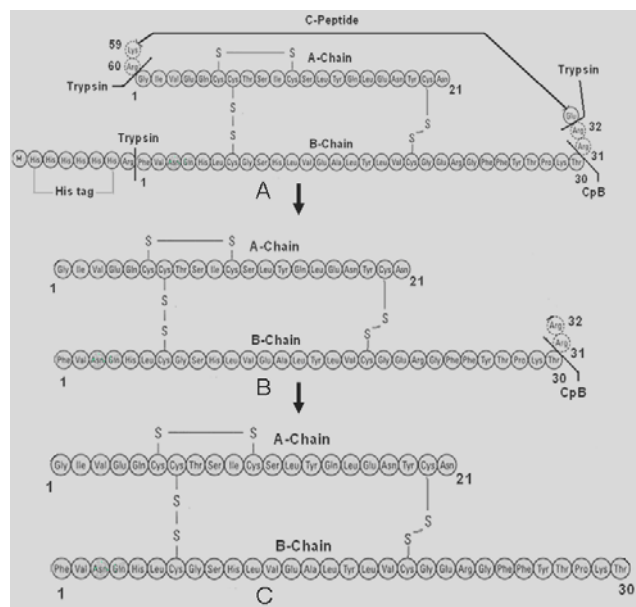
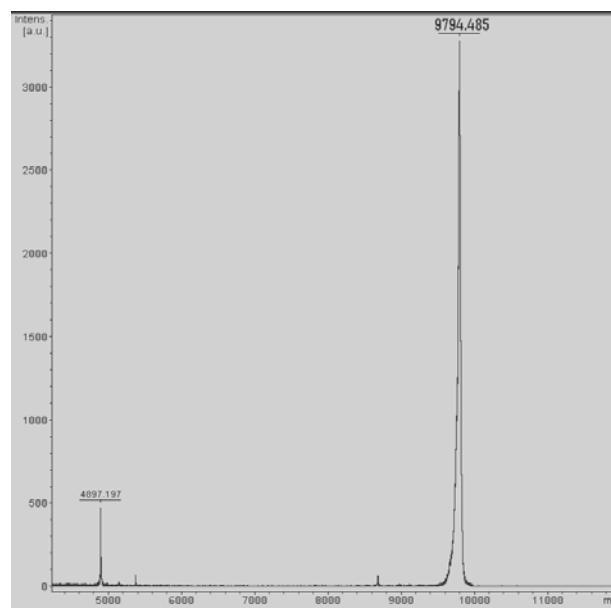


Fig. 5: Activation of recombinant River Buffalo proinsulin- [Met (His)₆ Arg-Proinsulin]. **A-** The primary structure of proinsulin derivative indicating the chain A, B and C-peptides with restriction sites for trypsin and CPB. **B-** The removal of N-terminal [Met (His)₆ Arg] and C-peptide except the Arg³¹ and Arg³² by the tryptic digestion. **C-** The removal of Arg³¹ and Arg³² by the action of CpB and liberation of insulin.

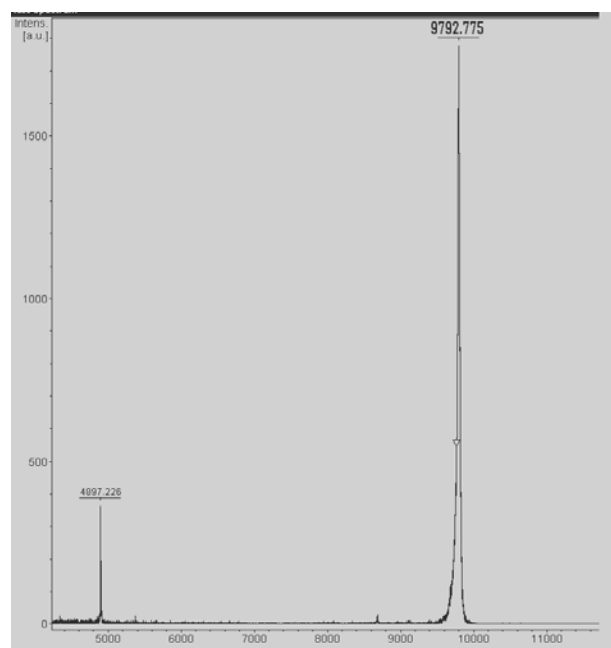
DISCUSSION

Carboxypeptidase-B was purified from *Bubalus bubalis* pancreas with selective ammonium sulphate precipitation and anion exchange chromatography with DEAE-Sephadex. The purified enzyme gave two bands on SDS-PAGE at 26 kDa and 9 kDa (fig. 1). Similar results have been reported by studies with human pancreatic CPB (Geokas *et al.*, 1975). The molecular weights of pancreatic carboxypeptidase B from porcine 36477 Da (Burgos *et al.*, 1991), ostrich 35,000 Da (Bradley *et al.*, 1996), bovine 34,000 Da (Reeck *et al.*, 1971), camel 31500 Da (Al-Ajlan and Bailey, 1999) are similar to the combined molecular weight of smaller and larger fragments of the purified enzyme. Our investigations have shown that the fragments are generated by proteolytic digestion of same polypeptide chain during the process of autolysis of pancreatic tissue. Our results have suggested that non-covalent interactions held the two fragments together after the restriction between Thr²⁰⁴ and Glu²⁰⁵ and the activity was retained (fig. 2). Despite of having a unique electrophoretic pattern with two fragments, the enzyme has shown kinetic properties like optimum pH (9.0), temperature (40°C) (fig. 4). Michaelis Constant K_M (30 μ M), turnover number K_{cat} (72 sec^{-1}) and specificity constant K_{cat}/K_M ($2.4 \times 10^5 \text{M}^{-1} \text{sec}^{-1}$) comparable with that of CPB reported from pig (Wolff *et al.*, 1962), camel (Al-Ajlan and Bailey, 1999), cow (McKay *et al.*, 1979), and

dogfish (Hajjou *et al.*, 1995). The enzyme has shown no activity with Hippuryl-L-Phe (substrate of carboxypeptidase-A).



(A)

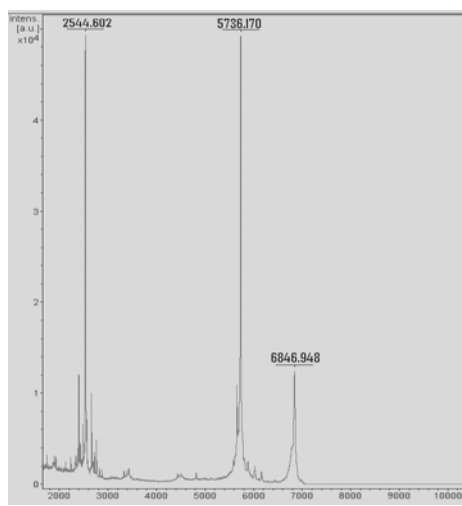


(B)

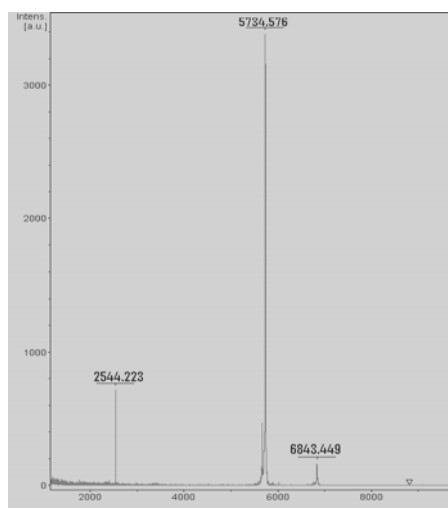
Fig. 6: MALDI-TOF analysis after the addition of CPB to the solution containing His-tagged derivative of River Buffalo proinsulin **(A)**-MALDI-TOF analysis of sample at 0 min after the addition of CPB. **(B)**-MALDI-TOF analysis of sample after 60 min. Theoretical molecular weight of recombinant proinsulin derivative is 9791.1Da as calculated from ProtParam.

Table: Enzyme activity, specific activity, percentage yield and fold purification of carboxypeptidase B at different stages of purification. One unit of enzyme is the amount of enzyme that catalyzes the conversion of one micromole of Hippuryl-L-Arg in one minute under the assay conditions used in the present study.

Purification Steps	Activity (Units)	Protein Content (g)	Specific Activity (Unit/mg)	Percentage Recovery	Fold Purification
Crude Extract	54000	32.0	1.69	100	1.0
30% Ammonium Sulphate Supernatant	51415	26.2	1.96	95.2	1.16
65% Ammonium Sulphate Precipitate	40800	11.8	3.46	75.0	2.1
Dialysate	39400	9.9	3.97	72.96	2.3
Anion Exchange Column	11340	0.094	120	21.0	71



(A)



(B)

Fig. 7: MALDI-TOF analysis of His-tagged proinsulin (100µg) after treatment with trypsin and CpB. **(A)**- Sample 5min after the addition of enzymes **(B)**- Sample 15min post treatment with enzymes. The proinsulin derivative, insulin and His-tagged insulin have theoretical molecular weights of 9791.1Da, 5733.5Da and 6843.0Da respectively.

The *in vitro* processing of proinsulin was carried out with trypsin and purified pancreatic CpB. Trypsin is a serine protease that cleaves the peptide chain at the carboxyl side of basic amino acids like lysine and arginine, except when either is followed by proline. Although the processing of proinsulin has been reported in previous studies (Donald *et al.*, 1971; Yang *et al.*, 1999; Markvicheva *et al.*, 2000), in the present study the process was monitored by MALDI-TOF analysis of peptides before and after enzyme catalyzed restriction. The MALDI-TOF monitoring of process has thrown light on the stepwise mechanism of proinsulin activation. Our study describes the first report on purification, structural and kinetic properties of pancreatic CpB from an unexplored species and has introduced an ms monitored procedure for the *in vitro* activation of proinsulin.

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