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AUTOLYSIS OF PROTEOLYTIC ENZYME IN METASTATIC BREAST CANCEROUS TISSUE

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Studies on autolysis of proteolytic enzyme like cathepsin revealed that the purified enzyme was autolysed by metastasis or due to absence of stabilizer. Crude tissue was extracted with sodium chloride buffer containing EDTA and Triton x-100. Extract was analyzed for total protein and protease activity. Protein/peptide was analyzed/purified using SDS polyacrylamide gel electrophoresis, ion exchange chromatography and reversed phase HPLC. Results indicated the autolysis during purification of enzyme. It is concluded that during and after purification suitable stabilizer of enzyme should be added. *In vitro* experiment indicated that *in vivo* (cancerous tissue), the imbalance of specific stabilizer/inhibitors of protease cause autolysis of enzyme and the resulting peptides cause the damaging effects.

Key words: Proteases, Chromatography, Breast cancer, Autolysis.

Introduction

The investigative efforts of great many scientists in both basic and clinical research have been devoted to understanding the biology of breast cancer. From laboratory studies of pathogenesis and metastatic potential of breast cancer, several protein products and genetic factors have been identified that may play important roles in the etiology and progression of breast tissue. Proteases are different to proteins. In comparison with proteins of similar size, proteases have smaller than average surface area, smaller radii of gyration and higher alpha densities. These findings imply that proteases are as a group, more tightly packed than other proteins. In structure, proteases have few helices and more loops. It is speculated that both high packing density and low alpha helical content co-evolved in proteases to avoid autolysis (Stawiski *et al* 2000). Staw (1994) found that autolysis of enzyme is due to the imbalance of the level of activators and inhibitors. Kumura *et al* (2000) found the autolysis of the protease by *Pseudomonas fluorescene* and observed that a pre-sequence of 13 amino acid residue was missing in the absence of specific inhibitor. Autolysis of proteolytic enzyme or proteases in metastatic breast tissue may result in short peptids.These peptides worsen the effects of cancer (Tariq 1992). We have therefore initiated to isolate the enzyme (any endopeptidase) from cancerous tissue and try to study its autolysis.

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Materials and Methods

Human breast tissue was obtained at surgery from surgical ward of Jinnah Hospital, Karachi. It was characterized histopathologically as malignant. Tissue was frozen immediately after surgery in liquid nitrogen and stored at 70°C until use.

*Extraction.*Human breast tissue (500 gm) was thawed at 4°C. It was rinsed with 0.15M sodium chloride (pH 3.8) containing 1mM EDTA and homogenized using homogenizer, Yamato Model K-41 in 0.15M sodium chloride buffer (pH 5.5) containing 1mM EDTA and 0.05% Triton X-100 to produce a fine suspension of the tissue. The homogenate was acidified to pH 3.8 with 1M HCl and incubated overnight at 4°C for autolysis. The homogenate was centrifuged at 3000g for 15min. Insoluble material was removed and supernatant was mixed with distilled chloroform in 3:1 ratio and centrifuged at 20,000g for 30 min. The upper layer was concentrated *in vacuo* and stored at 20°C (Toi *et al* 1996).

Protein estimation. Concentration of total protein and fractions of ion exchange chromatography (Amberlite IRC-120 and CM- cellulose) was estimated by Biuret method (Wooton and Freeman 1972) using bovine serum albumin as standard. The concentrated tissue extract and fractions of ion exchange chromatography was diluted and small aliquots were taken for analysis. The absorption was read on a Schmadzu spectrophotometer at 540nm.

Assay of endopeptidase activity. Endopeptidase activity of crude tissue extract and fractions of ion exchange chroma-

tography was assayed using azocasein as substrate. Activity units were calculated after measuring the absorbance of the reaction mixture at 440nm (Beynun and Bund 1993).

Electrophoresis. Slab gel electophoresis of crude tissue extract and fractions of ion exchange chromatography was performed in the presence of mercaptoethanol according to the method of Laemmli (1970).

Amino acid analysis. Purified fraction of reversed phase HPLC was hydrolyzed with 5.7N HCl for 24 h at 11°C. The sample was dried in vacuo and analyzed on automatic amino acid analyzer, Biotronic Lc 6001, Germany (Bollag *et al* 1996).

Column chromatography. Prefractionation of the crude extract was carried out using ion exchange chromatography. Amberlite IRC-120 column (2.5x23cm) was equilibrated with 20mM sodium acetate buffer (pH 5.0) containing 1mM EDTA and 0.5mM dithiotheritol. The sample was equilibrated overnight with the starting buffer. Flow rate was maintained at 1ml/min and fractions of 15 ml each were collected. The fractions corresponding to the first peak were pooled and concentrated *in vacuo.* A second peak was eluted with 0.5M Tris-HCl buffer (pH 8.0), containing 1mM EDTA and 0.5mM dithiothreitol. The concentrated fraction of first peak from Amberlite IRC-120 column was dialyzed against 20mM sodium acetate buffer (pH 5.0) containing 1mM EDTA and 0.5mM dithiothreitol and chromatographed on CM-Cellulose column (2.5x30cm), equilibrated with the same buffer. A sodium chloride concentration gradient of 0-40mM sodium chloride was established. Flow rate was maintainted at 0.53ml h^{-1} and fractions of 8.0ml each were measured at 280nm (Ethrington 1974).The fractions of each peak were pooled and concentrated *in vacuo*. The concentrated fraction of CM-Cellulose column was chromatographed on reversed phase HPLC using column Vydac C4. The elution was carried out with acetonitrile containing 0.05% TFA. Flow rate was maintained at 1ml min^{-1}. The absorbances were read at 280nm (Findlay and Geison 1989).

N-terminal sequencing. N-terminal sequencing of peptide was carried out manually using Edman Degradation method (Etheringon 1974).

Results and Discussion

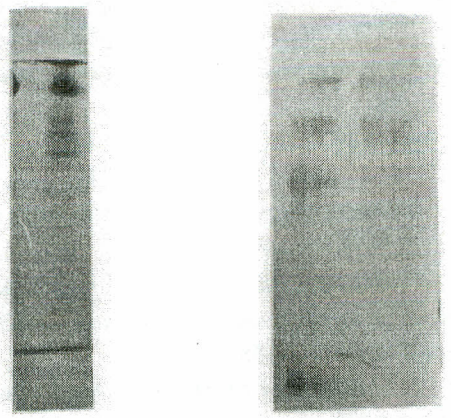
Total protein concentration of crude sample was found to be 5mg ml^{-1} whereas endopeptidase activity was 2.0 units ml^{-1}. Total protein concentration of fraction 1 and 11 (Amberlite IRC-120) was 1.4 mg ml^{-1} and 0.95mg ml^{-1} whereas endopeptidase activity of fraction of 1 and 11 was 3.0 units ml^{-1} and 0.5 units ml^{-1}. Total protein concentration of fractions 1,11,111,1V,V and V1 (CM-Cellulose) was 0.033 mg ml^{-1}, 0.022 mg ml^{-1}, 0.036 mg ml^{-1}, 0.25 mg ml^{-1}, 0.036 mg ml^{-1} and 0.025 mg ml^{-1} whereas endopeptidase activity was 0.4 units ml^{-1} in case of fractions 1 and 1V. No endopeptidase activity was observed in fractions 11, 111, 1V and V (Table1).

Electrophoresis of the crude tissue extract was carried out using 12.5% polyacrylamide gel. Prefractionation of crude tissue extract on Amberlite IRC-120 resulted in 2 peaks. Fractions of these peaks were pooled and concentrated *in vacuo*. These fractions were analyzed by 12.5% SDS polyacrylamide gel electrophoresis. Figure 1(a) shows the separation of proteins under denaturing conditions, Fig 1(b) shows electrophoresis of fractions whereas Fig (2) shows ion-exchange chromatogram of the crude tissue extract on Amberlite IRC-20.

The concentrated fraction of the first peak from Amberlite IRC-120 column was chromatographed on CM-Cellulose resulting in 6 peaks (Fig 3). Fractions of these peaks were pooled and concentrated in vacuo. These fractions were analyzed by 12.5% SDS polyacrylamide gel electrophoresis (Fig 4). The concentrated fraction of the 6th peak from CM-Cellulose was chromatographed on reversed phase HPLC resulting in 6 peaks. N-terminal sequencing checked purity of each peak. The purified fraction of the 4th peak gave single amino acid,

Table 1
Total protein and specific activity of endopeptidase

Parameters	Crude tissue exract	Fraction I (Amberlite)	Fraction II (Amberlite)	Fraction I&VI (CM-cellulose)	Fraction II,III,IV & V (RP-HPLC)
Total protein (mg ml^{-1})	5.0	1.4	0.95	0.033/0.25	0.022, 0.036, 0.25, 0.036
Endopeptidase activity (units ml^{-1})	2.0	3.0	0.50	0.40/0.40	0.00
Specific activity	0.4	2.1	0.50	12.0/16.0	0.00



1(a) 1(b)

Fig 1(a). Electrophoresis of crude extract.
Fig 1(b). Electrophoresis of fractions of Amberlite IRC-120.

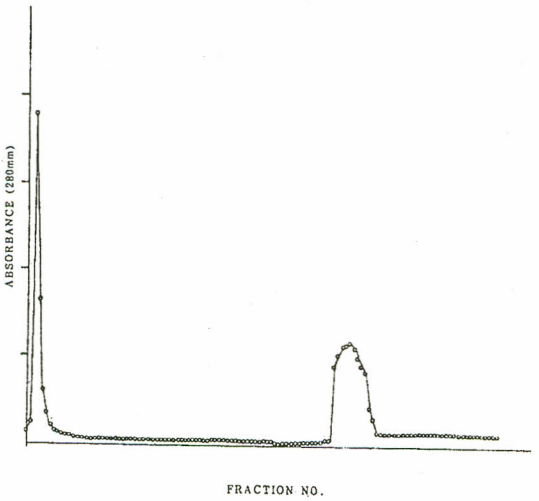


Fig 2. Ion exchange chromatography on Amberlite IRC-120.

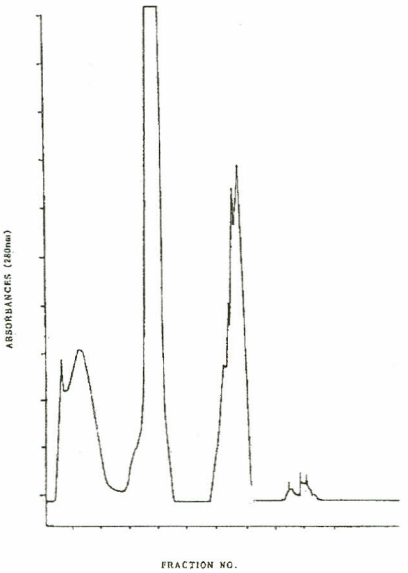


Fig 3. Unbound fraction of AM-IRC 120 on CM cellulose.

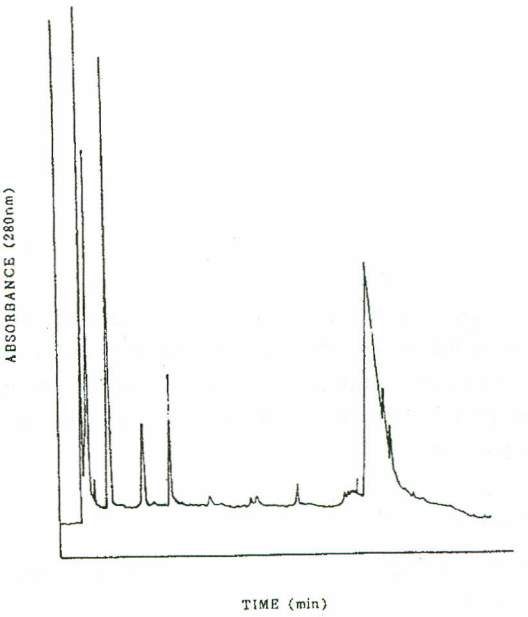


Fig 4. Reverse phase HPLC.

tyrosine. Mass of the peptide was found to be 660 dalton. N-terminal sequence was found to be YDLG..... (Tyr, Asp, Leu, Gly....). These sequences were matched with sequences of different endopeptidases and it was found that it matched with the sequence of transketolase (EC 2.2.1.1) which means that the matched sequence of amino

Table 2
Amino acid analysis of peptide from breast cancerous tissue

Amino acid	Concentration (n mol)	No of amino acid
Asp	0.6	1.0
Thr	N.D	-
Ser	N.D	-
Glu	N.D	-
Pro	N.D	-
Gly	427.9	428
Ala	N.D	-
Val	N.D	-
Cys	VL	-
Met	N.D	-
Ile	N.D	-
Leu	VL	-
Tyr	5.8	6.0
Phe	N.D	-
His	14.2	14.0
Trp	N.D	-
Lys	N.D	-

N.D, not determined; VL, Very low.

acid presents 293rd position. It means that a pre-sequence of 292 amino acid residue is autolysed. No antibacterial activity of this peptide was observed.

Conclusion

It is concluded that the purified peptide may be formed due to autolysis of enzyme as it did not exhibit any endopeptidase activity. The peptide may be of transketolase. Amino acid analysis and N-terminal sequencing of peptide revealed that the peptide was rich in glycine showing its neutral nature. In absence of any stabilizer or preservative the proteolytic enzyme may be activated in breast cancer (*in vivo*)and give proteolytic cleavage.

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