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Serine Protease Granzyme H Isolated from Lymph Nodes of Breast Cancer Patient

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Granzymes over expression is a market of Cytotoxic cell activation and should be further evaluated in patients with malignancies to delineate their potential value in predicting clinical outcome. Axillary lymph nodes contribute the main filter guarding against dissemination of breast disease. Granzyme H was isolated from lymph nodes by ammonium sulfate precipitation and technique of gel filtration chromatography.

Key words: Granzyme H, electrophoresis, chromatography, lymph nodes

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Introduction

Proteases are involved in many cellular and extracellular processes. Regulation of these enzymes is required to prevent abnormal tissue damage. In addition to controlling endogenous proteases, tissue need to be able to control proteases released by micro-organisms, inflammatory cells i.e. monocyte, macrophages, polymorpho nuclear leukocytes, mast cells and natural killer cells (Twining, 1994). Cytotoxic T lymphocytes and Natural killer cells are able to kill their target cells through synergistic action of perforin and granzymes resulting in distinctive nuclear changes typical of apoptosis (Quan *et al*., 1976). The uptakes of granzyme by nucleus take place at a range of basic pH (Trapani *et al*., 1996). Study demonstrates the selective binding of granzyme to several proteins present in target cells. The binding specificity is preserved when enzyme binding is performed in response of excessive competing protein including lysozyme and RNAase (Cationic species). Sub cellular fractionation of target cells shows that nuclear fraction contain granzyme binding reactivity. Granzyme interact with nucleolin (100 Kda), present in nucleus. This results in the proteolytic cleavage of nucleolin from 100-88 Kda and interaction of nucleolin with granzyme may be in the process of apoptosis (Pasternack *et al*., 1991). Granzymes over expression is a marker of cytotoxic cell activation and should be further evaluated in patients with malignancies to delineate their potential value in predicting clinical outcome (Later-Ravet *et al*., 1994). Axillary lymh nodes contribute the main filter guarding against dissemination of breast disease. Research data of Hindle (1990) indicates that 10-20 lymph nodes arranged as to trap metastasis at different levels in axilla.

Materials and Methods

Human lymph nodes were obtained at surgery, it was characterized histopathology as malignant and metastatic. Lymph node was frozen immediately after surgery in liquid nitrogen and stored at -70°C until use.

Extraction and Autolysis: Human lymph node (5.0 gm)_ was thawed at 4°C. It was rinsed with 10mM Tris-Hcl, buffer (pH 7.4) containing 1.5 mM EDTA, 0.5 mM DTT and 0.2mM phenylm ethylsulfonylfloride and homogenized (Homogenizer, Yamato Model K-41) with the same buffer. Homogenate was centrifuged at 1000 xg for 10 min. Insolubel material was removed and supernatant was centrifuged at 15,000 xv for 15 min. Precipitate was dissolved in 10 mM Tris-Hcl (pH 7.4) and
protein precipitated with ammonium sulfate (Dalet Fumeron, 1991).

Ammonium Sulfate Precipitation: Solid ammonium sulfate was slowly added (with concentration of 1M, 2M, 3M and 4M) and allowed to mix for 30 min. Protein precipitates were collected by centrifugation at 10,000 xg. Dissolve precipitate in 50 mM sodium acetate buffer (pH 6.5) and analyzed by electrophoresis (Bollag *et al*., 1996).

Column chromatography: Pre-fractionation of precipitate of 4M ammoniu m sulfate was carried out using Sephadex G-75 (1 x 12 cm). Sample was eluted with 50 mM sodium acetate buffer (pH 6.5) containing 10 mM EDTA and 0.5 % Triton x-= 100. Flow rate was maintained at 0.5 ml/5 min. Absorbencies were read at 280 nm. Fractions of 2nd peak containing protease activity were collected in vacuo and analyzed by electrophoresis (Bollag *et al*., 1996).

Protein Estimation: Concentration of total protein of crude extract and fractions of column chromatography was estimated by ultraviolet absorption on Shimadzu Spectrophotometer at 260, 280, and 320nm respectively (Bollag *et al*., 1996).

Protease activity: Enzyme activity was observed using 2.0% nutrient agar containing casein (microbiological assay).

Electrophoresis: Slab gel electrophoresis of crude tissue extract and fractions of column chromatography was carried out using 12% polyacrylamide gel in the presence of mercaptoethanol according to the method of Laemmli, 1970. Purity was confirmed by charge gel electrophoresis on 10% polyacrylamide gel. Calibration was achieved using a molecular mass kit. **Enzyme inhibitory assay:** It was reported that prednisone completely inhibited granzyme H, when incubated with granzyme H at 40°C for 1 hour (Cherin *et al*., 1996). It will be proved by electrophoresis.

Results

Protein content of crude extract of lymph nodes was found to be 4.4 mg/ml and Protease activity was found to be 0.5 units. Protein content of crude extract was precipitated with 1-4 M ammonium sulfate and their protein content was found to be 1.18, 1.45, 1.48 and 0.8 gm/100 ml. Whereas protease

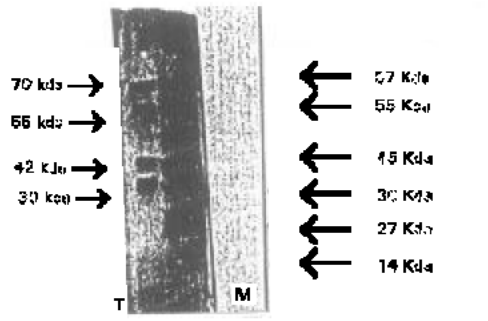

Fig. 1: SDS-PAGE of lymph node (precipitate with 4M ammonium sulfate) using 12% polyacrylamide gel (S = sample, M = Dye marker)

Fig. 2: Inhibitory activity of granzyme H is observed by 12% polyacrylamide gel (I = Inhibitor)

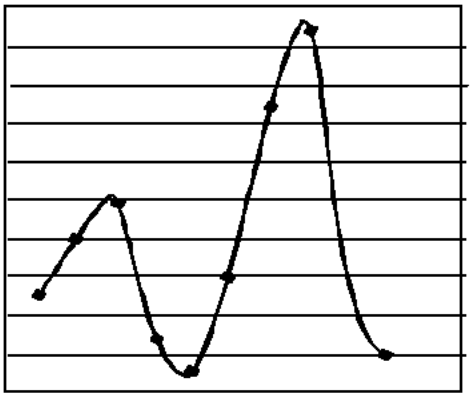

Fig. 3: Elution profile of lymph node extract precipitated by 4M ammonium sulfate on gel filtration using sephadex G-75

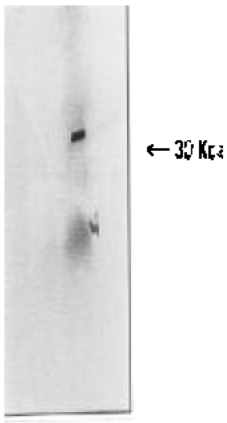

Fig. 4: Fraction of 2nd peak of sepadex G-75 was analyzed by 12% polyacrylamide gel under reducing condition

activity was observed only in crude extract precipitated with 3 and 4 M ammonium sulfate salt (protease activity was found by microbiological assay).

Electrophoresis of crude lymph nodes shows the protein of molecular weight range from 160-14Kda. Crude lymph nodes was precipitated with 4-0 M ammonium sulfate and analyzed by 12% polyacrylamide gel electrophoresis under reducing condition (Fig. 1). Inhibitory activity of granzyme H by prednisone was observed only in fraction precipitated with 4M ammonium sulfate (Fig. 2). Fraction precipitated by 4M ammonium sulfate was chromatographed on column of Sephadex G-75 resulted in 2 peaks (Fig. 3). Fraction of 2nd peak of column G-75 was analyzed by 12% polyacrylamide gel under reducing condition (Fig. 4). Results shows that purified granzyme have a molecular weight approximately 30 Kda. It is basic in nature as calculated by formula (Lys+arg/glu+asp).

Discussion

Serine proteases (granzymes) contained within the cytoplasmic granules of cytotoxic T cells and natural killer cells play a variety of roles including the induction of target cell apoptosis, breakdown of extracellular matrix proteins and induction of cytokine secretion by a bystander leukocytes. Different granzymes display proteolytic specificities that mimic the activities of trypsin or chymotrypsin, or may cleave substrates at acidic ("Asp-ase") or at long unbranched amino acids such as Met or "Met-ase" (Edward *et al*., 1999).

Results shows that purified granzyme have a molecular weight approximately 35 Kda. (Edward *et al*., 1999) who reported that molecular weight of granyzme H is 32 Kda. It is basic in nature as calculated by formula (Lys+arg/glu+asp). Maclover *et al*. (1999) reported that human granzyme H is a neutral serine protease, that is expressed predominatnly in the lymphokine-activated killer (LAK/natural killer (NK) compartment of the immune system. We also observed that granzyme is inhibited by prednisone. Cherin *et al*. (1996) confirmed that prednisone inhibit granzyme. Hence further research is needed to study the role of granzyme H in breast cancer metastasis which usually occurs through lymph nodes.

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