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ION EXCHANGERS: A USEFUL TOOL FOR SEPARATION AND SIMULTANEOUS PURIFICATION OF LYSOSOMAL CYSTEINE PROTEINASES, CATHEPSINS B, H AND L.

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ABSTRACT: Lysosomes of mammalian tissues contain a number of cysteine proteinases such as Cathepsins B, H and L, which participate in intracellular protein degradation and are involved in various pathological disorders. These proteinases have been purified to ~ 1600 fold with ~25% yields from goat liver. These Cathepsins having similar molecular weight (~25,000 Da) were obtained as a single pool after initial purification steps i.e. homogenization of acetone powder, acid autolysis at pH 4.0, 30-70 % $(NH_4)_2SO_4$ fractionation and molecular sieve chromatography on Sephadex G-100, were simultaneously separated and purified on exchange chromatographies. Cathepsin L was completely separated and purified from Cathepsin B & H at CM- Sephadex C-50 column at pH 5.6 and was eluted as bound protein at 0.62 M NaCl with a purification fold ~1629 and 22 % yield. Cathepsin B was eluted as bound protein in ~1545 fold with ~45% yield at 0.40 M concentration. Complete separation of Cathepsin H from Cathepsin B was achieved on DEAE-Sephadex A-50 at pH 6.0 where the former was obtained as unbound protein and the latter; Cathepsin H thus obtained was purified to ~ 1601.11 fold with ~29.18% yield. Cathepsin B, H & L were thus purified using ion-exchange chromatography.

INTRODUCTION: Mammalian tissues contain a number of lysosomal proteinases [1-4] which belongs to papain super family. Besides their role inside lysosomes, these cysteine proteinases also degrade proteins outside lysosomes [5]. The cathepsins alone are involved in protein breakdown in lysosomes, antigen presentation, proteolytic processing of proenzymes and prohormones, fertilization, cell proliferation, differentiation and apoptosis [6-8]. Cathepsins B (EC 3.4.22.1), Cathepsins H (EC 3.4.22.16) and Cathepsins L (EC 3.4.22.15), are lysosomal proteolytic enzymes belonging to the cysteine protease family.

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They are widely distributed in almost all mammalian cells, being mainly responsible for intracellular protein degradation [9] and protein turnover [10-13]. When secreted lysosomal cysteine proteinases result in pathological conditions, they are observed to be involved in a number of diseases such as muscular dystrophy [14], malignant melanoma [15-16], cancer [17-19], rheumatoid arthritis [20], osteoarthritis[21-22], inflammatory and bone and joint disorders [23], Alzheimer's disease [24-25], multiple sclerosis [26] and pancreatitis [27].

In many of these diseases lysosomal enzymes were found to be present in extracellular / extralysosomal environment in the proforms, which are substantially more stable than the mature enzymes [28]. The physiological, pathological role of these important protein processors necessitates their identification, isolation, purification and characterization at the required site. The present work focuses on Cathepsins B, H & L having

similar molecular weight [29-31], how ion exchangers can be efficiently employed for simultaneous purification of Cathepsins B, H & L from a single source i.e. goat liver.

MATERIALS AND METHODS:

All the chemicals were of analytical grade. Fast Garnet GBC (*o*-aminoazotoluene diazonium salt, α-N – benzoyl - D, L-arginine - 2-naphthylamide (BANA), Z-Arg-Arg-NNapOMe, Z-Phe-Arg-NNapOMe and Leu-2NNap were purchased from Bachern Feinchemikalien AG, Switzerland. Sephadex G-100, CM-Sephadex C-50 and DEAE-Sephadex-A-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The protein sample was concentrated using Amicon stirred cells with YM 10 membrane under nitrogen pressure of 4–5 psi. The source of enzyme was fresh goat liver obtained from local slaughter house. Glass double distilled water was used in all the experiments.

Enzyme Assay:

Cathepsin B activity was determined using BANA or Z-Arg-Agr-NNapOMe as substrate [29] at pH 6.0. The activity of Cathepsin H was assayed as described previously [30] using Leu-2-NNap as substrate at pH 7.0 and also by BANA at pH 6.5. Cathepsin L activity was assayed [31] using Z-Phe-Agr-NNapOMe at pH 6.0.

Protein assay:

The acid soluble proteins were quantitated in the supernatant using Bradford method [32].

Preparation of acetone powder:

Fresh goat liver was washed thoroughly with cold saline solution to remove blood capillaries and was minced properly with ten volumes of chilled acetone in a mixer cum grinder to disintegrate the tissue. The precipitate thus obtained, were filtered, using a Buchner funnel. The residues were resuspended in three volumes of chilled acetone, homogenized, and filtered. Finally, the filtrates were collected and a stream of nitrogen was flushed through it and dried in vacuum over concentrated sulphuric acid and the acetone powder was stored over anhydrous calcium chloride at 4°C until use.

Separation and purification of Cathepsins B, H and L: All the purification steps were carried out at 4°C unless otherwise stated. The initial steps of purification i.e., Goat liver acetone powder homogenization in cold 0.1 M sodium acetate buffer pH 5.5 containing 0.2 M NaCl and 1mM EDTA, Acid-autolysis at pH 4·0 and 30-80% ammonium sulphate fractionation. Fractionation of proteases based on molecular weight on Sephadex G-100 column chromatography according to the method described by Kamboj et.al[29-31]. 2-Mercaptoethanol (2mM) was included in all these steps, except during acetone powder preparation. 50 ml of enzyme solution was applied to a column of Sephadex G-100 equilibrated with 0.1M sodium acetate buffer pH 4.76 containing 0.2mM NaCl, 1mM disodium EDTA.

The column was eluted with the same buffer-EDTA solution at a flow rate of 40 ml\h, and the fractions of 10 ml were collected and stored at 4°C till the absorbance of effluent buffer at 280nm was nil against the column buffer. The enzyme activities were measured using BANA. Thereafter BANA active fractions were analysed for Z-Arg-Arg-NNapOMe, Leu-2-NNap and Z-Phe-Arg-NNapOMe hydrolysing activities to check the presence of Cathepsin B, H and L **(Fig. 1).**

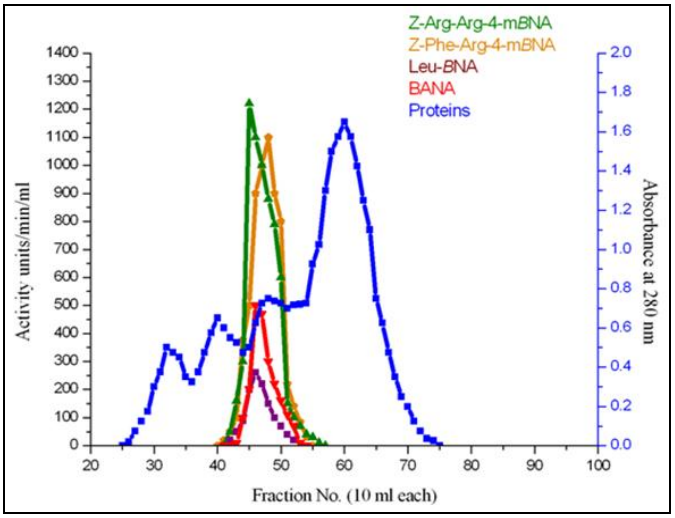

FIG.1: MOLECULAR SIEVE CHROMATOGRAPHY ON SEPHADEX G-100 COLUMN SHOWING PROTEIN, BANA, Leu-βNA, Z-Arg-Arg-4-m βNA, Z-Arg-Arg-4-m βNA HYDROLYZING ACTIVITIES.

The fractions active against BANA, Leu-2-NNap Z-Arg-Arg-NNapOMe and Z-Phe-Arg-NNapOMe obtained from Sephadex G-100 column were pooled, concentrated and dialysed against 20mM sodium acetate buffer pH 5.6 containing 1mM

disodium EDTA and 2mM 2-mercaptoethanol for 24 h. Cathepsin B, H and L having molecular weights in the same range[29-31] (~25,000) were eluted as one peak from Sephadex G-100 column and further purified using ion-exchange chromatography.

Ion Exchange Chromatography on CM-Sehadex C-50:

The dialyzed enzyme sample was applied to a column of CM-Sephadex C-50 equilibrated with 20 mM sodium acetate buffer pH 5.6 containing 1 mM disodium EDTA and 2mM 2-mercaptoethanol. The column was washed with same buffer-EDTA-mercaptoethanol solution. The fractions of 5ml were monitered for their protein content by measuring the absorbance at 280nm. When the absorbance of effluent buffer for unbound protein at 280nm was found nil, the bound proteins were eluted with a linear NaCl (0.0-0.8M) gradient and the fractions of 5ml were collected and were analysed for their protein content by measuring the absorbance at 280nm.

The unbound and salt-gradient eluted fractions were analyzed for their activities against BANA, Leu-2-NNap, Z-Arg-Arg-NNapOMe and Z-Phe-Arg-NNapOMe. The Z-Arg-Arg-NNapOMe and Z-Phe-Arg-NNapOMe hydrolyzing activities were pooled separately. The fractions containing both BANA as well as Leu-2-NNap hydrolyzing activities were pooled together and processed for separation of Cathepsin B from H **(Fig. 2).**

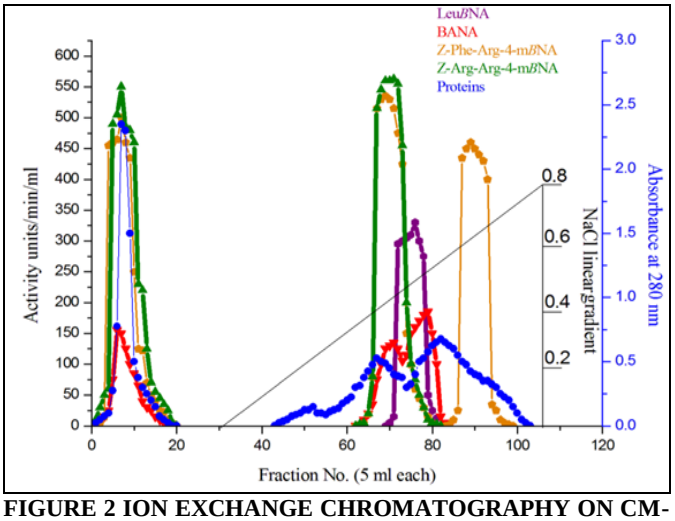

FIGURE 2 ION EXCHANGE CHROMATOGRAPHY ON CM-SEPHADEX C-50 COLUMN SHOWING PROTEIN, BANA, Leu-βNA, Z-Arg-Arg-4-m βNA, Z-Arg-Arg-4-m βNA HYDROLYZING ACTIVITIES

Anion Exchange Chromatography on DEAE-Sephadex A-50:

The above obtained combined pool of Cathepsin B and Cathepsin H were dialyzed against 20mM sodium phosphate buffer pH 6.0 containing 1mM disodium EDTA and 2mM 2-mercaptoethanol for 24h and were applied on DEAE-Sephadex A-50 column equilibrated with 20mM sodium phoshphate buffer (pH 6.0) containing 1mM disodium EDTA. After removing unbound proteins active against Leu-2-NNap, the fractions of bound proteins were eluted with NaCl linear (0.0-0.5M) gradient and were analysed for their activities against Z-Arg-Arg-NNapOMe **(Fig. 3).**

Z-Arg-Arg-NNapOMe hydrolyzing activity obtained after CM-Sephadex C-50 and DEAE-Sephadex A-50 were pooled together. The separated pools of Cathepsins B, H and L were pooled, concentrated to 15 ml and dialyzed against 50 mM sodium acetate buffer pH 4.6 containing 1 mM disodium EDTA, 0.2M NaCl were concentrated and stored at 4°C for further use. The preparations of Cathepsins B and H contained 0.1mM $HgCl_2$ whereas in cathepsin H 2mM 2-mercaptoethanol was added.

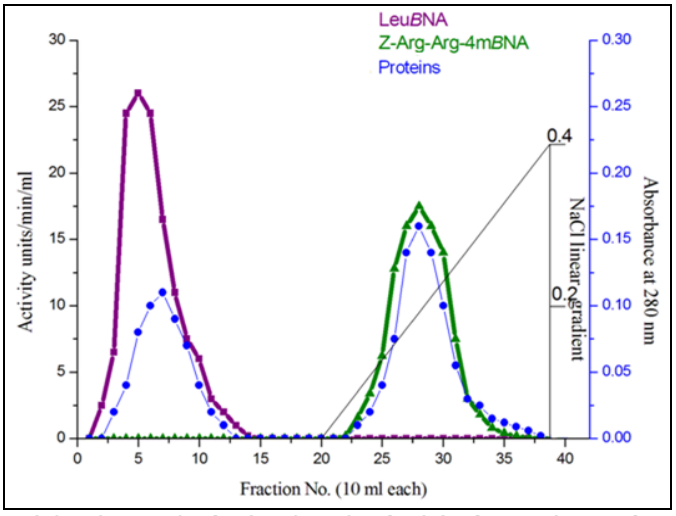

FIG.3: ION EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX A-50 COLUMN SHOWING PROTEIN, Leu-βNA, Z-Arg-Arg-4-m βNA HYDROLYZING ACTIVITIES.

RESULT AND DISCUSSION: Fig. 1 shows the elution profile of different enzymes on Molecular sieve chromatography on Sephadex G-100 column after ammonium sulphate fractionation. It helped in separation and isolation of Cathepsins B, H and L on the basis of their molecular weight (~ 25000)

from cathepsin D and DPPI. The results correlate with earlier work [29-31, 34.] Some workers [36-41] didn't include this step at an early stage of purification and hence could not accomplish simultaneous isolation of these proteases. Further fractionation of Sephadex G-100 pool on cation exchange chromatography at pH 5.6 resulted in complete separation of cathepsin L from B and L **(Fig. 2).** Cathepsin B was eluted as bound protein at 0.62M NaCl exclusively as Z-Phe-Arg-NNapOMe hydrolyzing activity. The separation of cathepsin L from cathepsin B and H on CM-Sephadex cation exchanger has earlier been reported [42-43]. Cathepsin B was present in unbound (~ 40%) as well as in bound fractions (~ 60%) as reported45-46 at pH 5.0. the bound Cathepsin H was eluted at 0.48M NaCl alongwith Cathepsin B.

Although at cation exchanger Cathepsin H showed a tendency to separate as indicated by a shoulder peak of BANA having Leu-2-NNap activity, complete separation of Cathepsin B and H was achieved on DEAE-Sephadex A-50 at pH 6.0, where Cathepsin H was eluted unbound and Cathepsin B was eluted at 0.2M NaCl. Earlier studies also report the separation of Cathepsin H from B on DEAE-Sephadex A-50[42-44]. Further purification of Cathepsin H and L was done on organomercuric affinity column to extensive inactivation of the enzymes.

Table 1 depicts the purification factor achieved and % yield obtained after each step of purification. The % yield obtained in all the three enzymes ranges between ~25% whereas ~1600 fold purification was achieved in all the three proteinases. The results are quite encouraging in comparison to earlier reports[37-38] in case of Cathepsin H. these workers have not reported simultaneous separation and purification of Cathepsins B, H and L. For the first time, cation and anion exchangers have been successfully used for simultaneous purification of three cysteine proteinases. We are working in the identification of small molecular weight compounds as inhibitors to endogenous proteolytic activities [46-51].

TABLE 1: PURIFICATION OF CATHEPSINS B, H AND L

S.No.	Purification Steps	Cathepsins B		Cathepsins H		Cathepsins L	
		Purification Factor	% Yield	Purification Factor	% Yield	Purification Factor	% Yield
1.	Crude Extract Supernatant, S_1	1.0	100.0	1.0	100.0	1.0	100.0
2.	Acid Fractionation Supernatant, S_2	1.75	99.31	1.40	79.84	1.72	98.52
3.	30-80% $(NH_4)_2SO_4$ Pellet, P_4	30.65	97.52	22.92	73.30	30.53	97.86
4.	Sephadex G-100 Pool	69.61	75.61	61.78	67.61	68.51	74.58
5.	CM-Sephadex C-50 Bound Pool	1545.99	45.71	1154.61	55.08	1629.96	22.22
7.	DEAE-Sephadex A-50 Pool	-	-	1601.11	29.18	-	-

The results presented here are of a single enzyme preparation that resulted in simultaneous separation and purification of Cathepsin B, H and L using different steps as detailed above. Cathepsin B, H and L activities were assayed using BANA, Leu-2-NNap, Z-Phe-Arg-NNapOMe at pH 6.0, 7.0 and 6.0, respectively. Cathepsin B, H and L has emerged out as targets for development of anticancer drugs. Therefore it becomes relevant to evaluate inhibitory potency of compounds of different classes known to possess anticancer activities on these purified enzymes. The enzymes separated using these steps have been used for studies of different compounds.[52-57]

CONCLUSION: From the literature study, it is clear that cathepsin B, H and L are important targets in cancer and other pathological disorders such as arthritic conditions and tissue generative disorders and the development of selective inhibitors to these enzymes are a rational approach in the search for chemotherapeutic agents. In this paper, we have reported simultaneous separation and purification of Cathepsins B, H and L using cation and anion exchangers.

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