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Studies on Brain Hydrolytic Enzymes and their Endogenous Protein Substrates

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The complete spectrum of proteolytic activity in the goat brain homogenate was determined by autolysis utilising only endogenous proteins as substrates. Three main peaks I, II and III of proteolytic activity were centred at pHs 3.5, 5.0 and 7.0, respectively. Inclusion of pepstatin, a specific inhibitor of cathepsin D, in assay mixtures shows that 70% of the activity in peak I is mainly due to this protease. The peak II consisted of thiol proteases like cathepsin B, L and BANA-hydrolase. This was shown by activation of the proteases in this peak by dithioerythritol and EDTA (thiol activators). When leupeptin, a specific inhibitor of cathepsin B and L was used, only 60% of the activity in peak II was abolished indicating thereby the presence of leupeptin-insensitive thiol proteases in addition to the presence of cathepsin B and L. The peak III contains proteases acting at neutral and alkaline pHs. The subcellular fractionation of the brain homogenate into nuclear, mitochondrial-lysosomal and soluble fractions confirmed that most of the activity in peaks I, II and III can be attributed to lysosomal proteases.

The central nervous system contains bioactive peptides in picomolar quantities which act as possible neurotransmitters, hormones and modulators of electrophysiological and secretory processes in neurons[1]. The involvement of these neuropeptides in the control mechanism has generated tremendous interest in the field of neurochemistry but has so far alluded proper understanding. In a recent review, Marks[2] highlighted the possible involvement of brain proteases vis-a-vis these bioactive peptides. It has been hypothesised that the proteases which are present inside the cell may be involved in the generation/inactivation of these bioactive peptides. A literature survey reveals that the work on brain proteases relates mostly to cathepsin D[3,4] (EC 3.4.23.5) acting at highly acidic pH, to proteases acting at near neutral pHs[5] and to a few neutral and alkaline proteases[6]. Some of these proteases have been purified partially or to apparent homogeneity and their substrate specificities have been studied utilising denatured proteins or chromogenic synthetic analogs as substrates. Unfortunately, all these studies have utilised exogenous (from outside the cell) protein substrates like haemoglobin, casein, azocasein and bovine serum albumin.

Bradley and Whitaker[7] have recently shown that purified brain cathepsin B (EC 3.4 22.1) degrades myelin basic protein isolated from the brain itself thereby deriving a good correlation vis-a-vis brain proteases and brain proteins as substrates. However, such a hypothesis presupposes that the hydrolytic enzynes stacked mainly in the lysosomes will have to come out, i.e. lysosomes have to be damaged, representing a diseased state. Kalnitsky *et al*[8]., while working with rabbit lung showed that in an established rabbit model of acute hypersensitivity pneumonitis, a chronic diseased state, the total and specific activities of cathepsin B, lysosomal carboxypeptidase B (EC 3.4.18.1), dipeptidylaminopeptidase I (EC 3.4.14.1) and cathepsin D increased tremendously. There are still other cases where the proteases show increased/decreased activities in the diseased conditions. But the vital question as to what are the natural substrates of these proteases under the *normal conditions*, still remains unanswered.

It might be highly unrealistic to draw conclusions about the specificities of proteases present in brain, based upon their hydrolytic action on such substrates which they may never encounter in their natural environment, i.e. inside the cell. The present study is the sole attempt to identify the existence of *endogenous protein substrates* for intracellular proteases in brain. It was done by allowing the proteases to digest the protein substrates present in the cell in the same environment as existed inside the cell, i.e. by autolysis. No additional exogenous proteins were added as substrates. The present study also deals with the subcellular localisation of proteases involved in the autolytic digestion of the native proteins.

Results and Discussion

Protease activity profile at various pHs: The brain homogenate contains large amounts of protein which may affect the final pH of the incubation mixture, hence the method was standardised by conducting trial experiments to observe how much shift in the pH values of buffer occurs by the addition of known volume of the homogenate. Based on these, the universal buffer in 12 tubes was adjusted by careful addition of 1*N* HCl or 1*N* NaOH to the following pHs: 1.35, 1.60, 1.70, 2.05, 2.78, 3.90, 5.45, 6.10, 6.85, 7.50, 8.00 and 8.75. Now 10% enzyme homogenate (4 ml) was mixed with the buffer (4 ml) at each of the pHs

listed above. This resulted into 5% homogenate, called assay mixture at each pH. From each of the 12 tubes above, the assay mixture (2 ml) was taken in duplicate tubes and incubated immediately at 37° for 3 h. The assay mixture (2 ml) at each pH was used as a blank where TCA was added immediately. The remaining assay mixture at each pH was utilised for measuring the actual pH values of the assay mixtures and were found to be 2.27, 3.27, 3.55, 4.13, 4.51, 4.83, 5.36, 5.76, 6.28, 6.78, 7.10 and 7.70 respectively. The reaction was stopped by the addition of TCA and the proteolytic activity was measured as described in 'Experimental'. The results depicted in Fig. 1 represent one typical experiment out of four such experiments performed at different times and with different brain homogenates. It is clear that the brain homogenate contains protease activity over the entire pH range 2.0–8.0. Experiments were also conducted up to 10.0 but no activity was obtained beyond pH 8.0.

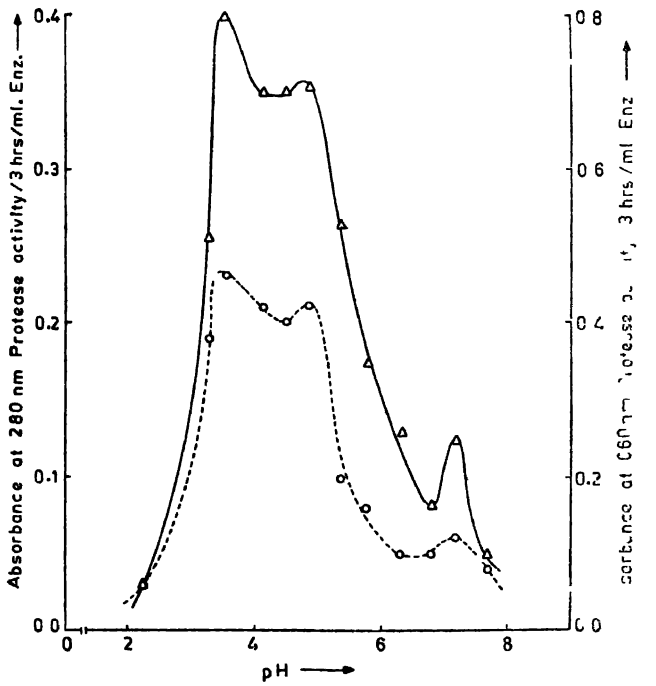

Fig. 1. Proteolytic activity of the brain homogenate at various pHs : after autolysis for 3 h at 37°, TCA-soluble peptides were estimated at (O) 280 or (Δ)660 nm.

It has been shown here for the first time that the endogenous proteases of brain tissue do have their substrates among the endogenous proteins of the same tissue. It may be emphasised that no additional exogenous proteins were added as substrates. The pH optima for the degradation of these endogenous proteins centred around pH 3.5, 5.0 and 7.0 (Fig. 1). By using a combination of exogenous substrates like haemoglobin and casein[9], and haemoglobin, azocasein and casein[10], it has already been established in this laboratory that the brain homogenate contains proteases which are active over the entire pH-range. In respect of these alien exogenous substrates, the endogenous proteases were more active around pH 3.5–4.0, 4.5—5.5 and 8.0—9.5. In the light of the reported value of 6.34 for the intralysosomal pH[11], it can be expected that under normal circumstances, these lysosomal proteases have no action on the endogenous protein substrates. However, the significance of these pH optima can be visualised in the light of the fact that an ATP-dependent proton pump[11,12] can bring and maintain the pH inside the lysosomes to acidic values. This kind of arrangement can act as a feedback mechanism to control the generation and degradation of bioactive peptides after proper signals are received by the lysosomes.

After demonstrating the presence of endogenous proteases and their endogenous protein substrates, it was thought proper to localise this protease activity. For this purpose, three subcellular fractions, namely, nuclear, mitochondrial-lysosomal and cytoplasmic (soluble) fractions were obtained as detailed in the 'Experimental'. Out of these, the latter two fractions were lysed with Triton X-100 and their protease activity was measured by autolysis. These results (Fig. 2) clearly indicate that most of the protein hydrolysing activity of

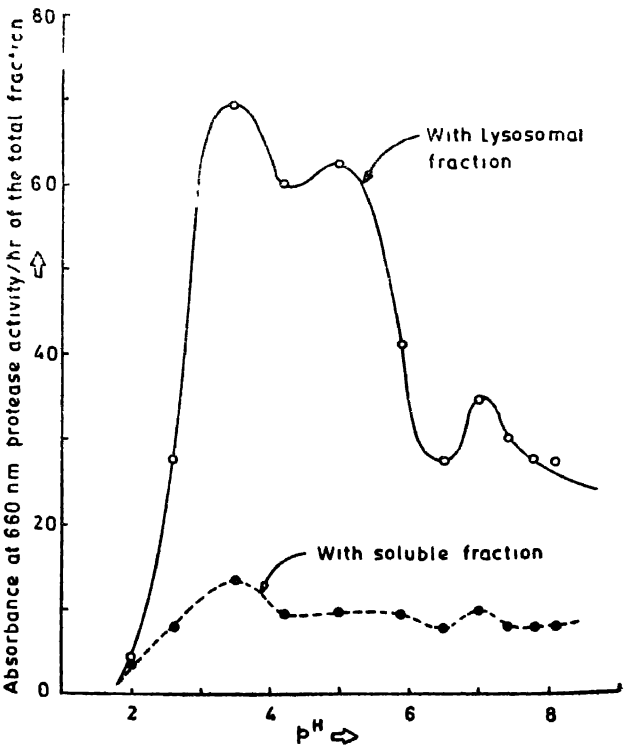

Fig. 2. Protease activity in the brain subcellular fractions : The mitochondrial-lysosomal and soluble fractions in 0.25*M* sucrose were homogenised separately incorporating Triton X-100 at 0.2% final concentration (v/v). The protease activity of these homogenates was measured by autolysis at various pHs.

brain tissue resides in the lysosomes. It is also clear that the hydrolytic enzymes residing in the lysosomes find suitable endogenous protein substrates within the lysosomes itself, and that the lysosomes contain proteases capable of acting over a wide range of pH including the very very acidic to slightly basic pH. Quantitative differences apart, there are present inside the lysosomes, the protein substrates for all these proteases. The bovine brain lysosomal hydrolase, cathepsin D[4], in a purified form has been reported to be capable of degrading bioactive substances like somatostatin (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys) and substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2). The protease activity in the soluble fraction is negligible. Nuclear fraction was not processed because it contained many unbroken cells which would have given rise to lysosomes on lysis.

Identification of proteases in activity peaks: After amply demonstrating through these autolytic studies that (i) brain homogenate contains proteolytic enzymes active over almost the entire pH range, (ii) suitable endogenous protein substrates are readily available to them, (iii) most of the proteases responsible for the protease activity can be localised in the lysosomal fraction establishing their lysosomal origin, (iv) the endogenous protein substrates also reside in the lysosomes and (v) only negligible amount of protease activity originates from the cytosolic fraction, it was thought appropriate to broadly identify the proteases responsible for such hydrolytic activity particularly in the 3 peaks of Fig. 1. There can be two ways to tackle this problem : (i) to separate and purify various hydrolytic enzymes present in the brain tissue and then study their action on endogenous protein substrates at various pHs and (ii) to use known and specific activators/inhibitors of certain well-characterised hydrolases and study the extent of activation/inhibition caused in the activities of such hydrolases. Towards this end, pepstatin, isovaleryl-L-valyl-L-valyl-AHMHA-L-alanyl-AHMHA (AHMHA is 4-amino-3-hydroxy-6-methylheptanoic acid) a very strong and specific inhibitor of cathepsin D[18] was included in the assay mixtures at final concentration of 1 μm, sufficient to block the activity of this protease completely. The results are shown in Fig. 3. Evidently, about 70% hydrolytic activity of peak I at pH 3.5 is lost without any effect whatsoever on the other two peaks demonstrating thereby that most of the activity in peak I is due to the acid-acting hydrolase, cathepsin D. This protease has been purified and characterised from brain tissue and has been shown to degrade exogenous proteins[3,4]. The present study shows the existence of endogenous protein substrates in brain for this protease. The residual activity in peak I may be due to the combination of several other proteases insensitive to pepstatin including significant contribution from cathepsin B and α-*N*-benzoyl D,L-arginine-β-naphthylamide-hydrolase/cathepsin *H* (EC 3.4.22.16)-type enzymes. Though the optimum pH for cathepsin B and BANA-hydrolase[14] with respect to synthetic substrates is around pH 6.0, yet Singh *et al.*[15] demonstrated that purified lung cathepsin B and BANA-hydrolase degrade collagen substrate maximally between pH 3.0 and 4.0. Other workers also reported that cathepsin B degrades haemoglobin[16], collagen[17] and cytosolic proteins[18] between pH 3.5 and 4.5.

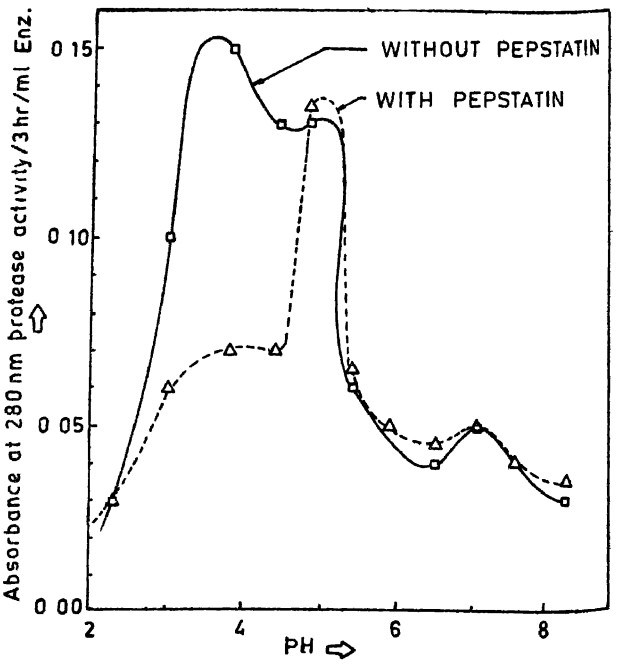

Fig. 3. Protease activity of the brain tissue at various pHs in presence and absence of pepstatin ; pepstatin at a final concentration of 1 μM was incorporated in the assay mixtures before measuring the activity by autolysis.

The identification of proteolytic enzymes in peak II (centred at pH 5.0) involved the use of DTE+EDTA, known activators of thiol proteases and when used at concentrations of 2 and 1 mM, respectively, they enhanced the activity present in peak II by 1.5-fold but had no effect whatsoever on other two peaks. Also, this activity in peak II was completely abolished when 1 mM PCMB, a wide spectrum inhibitor of thiol proteases, was included in the assay mixture (data not shown). The above two experiments confirm the presence of thiol proteases in peak II. For further identification, when leupeptin (acetyl-L-leu-L-leu-L-argininal), a specific inhibitor of cathepsin B[18] was included in the assay mixtures at a final concentration of 1 μM, about 60—70% activity was abolished (Fig. 4), indicating major contribution of cathepsin B and L (EC 3.4.22.15) in this protease peak. The remaining activity may be due to other thiol proteases like cathepsin H/BANA-hydrolase which are insensitive to this specific cathepsin B

inhibitor as was demonstrated by Singh and Kalnitsky[14,19] in case of rabbit lung BANA-hydrolase. To remove any ambiguity about the conclusions that the protease peak II of Fig. 1 consists mainly of leupeptin-sensitive thiol proteases cathepsins B and L, another experiment

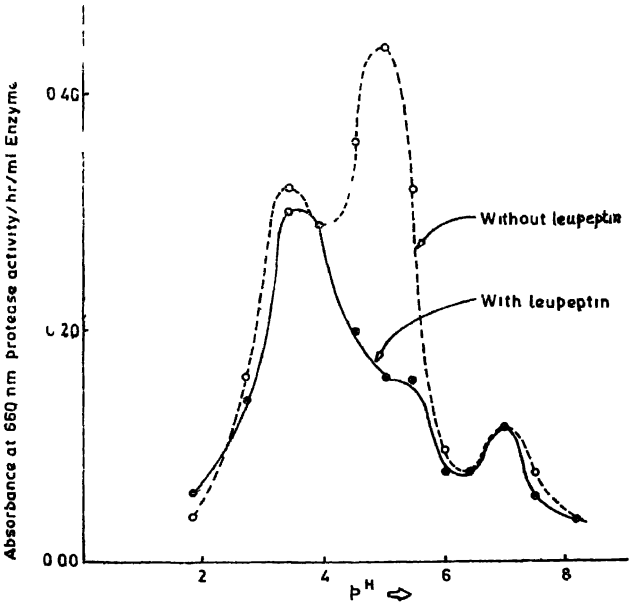

Fig. 4. Protease activity of the brain tissue at various pHs in presence and absence of leupeptin ; leupeptin at a final concentration of 1 μ *M* was incorporated in the assay mixtures before measuring the protease activity by autolysis.

was designed. For this purpose, one part of the freshly prepared brain homogenate was autolysed at various pHs without any additions at 37° for 3 h to obtain the usual protease profile. The pH of the second part was raised to 7.1 and this homogenate was incubated for 2 h at 5° at this pH. When this alkali-treated homogenate was autolysed at 37° at various pHs, the peak II was clearly abolished (Fig. 5). These results show that cathepsins B and L are inactivated by exposure to alkaline pH and hence no activity at slightly acidic pH of 5.0 (purified goat brain cathepsins B and L were irreversibly inactivated[20] by exposure to alkaline pH of 7.1). It is pointed out here that the protease activity profile in the highly acidic and basic range is unchanged because cathepsin (the main protease in peak I) and alkaline protease(s) are not inactivated by exposure to pH 7.1. However, when the purified goat brain cathepsins B (1 μg) and L (0.35 μg) were added to the assay mixtures at various pHs prepared for autolysis from the alkali-exposed homogenate and then autolysed at 37° for 3 h, it was found that the protease peak II of Figs. 1 and 5 was restored (Fig. 5). This experiment demonstrated clearly that the protease peak II is mainly due to leupeptin-sensitive thiol proteases cathepsins B and L. It also discounts any contribution in this peak coming from endogenous activators/inhibitors. Any bacterial protease action is also discounted because if it were so then there would be no difference in the protease activity profile during autolysis of the untreated and alkali treated brain homogenate.

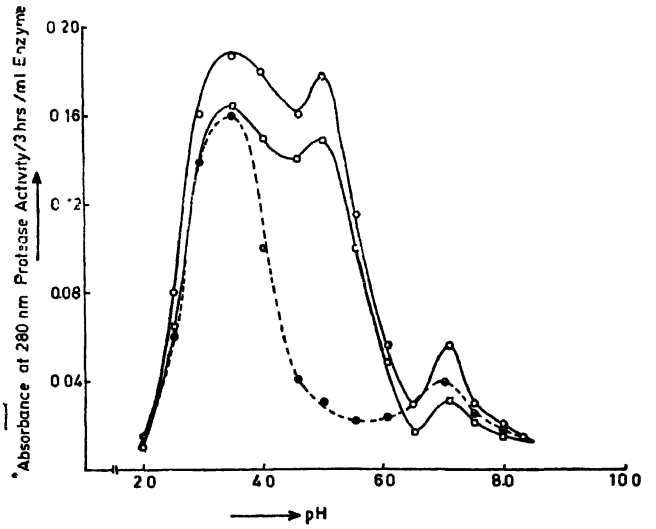

Fig. 5. Protease activity of brain homogenate, alkali-treated brain homogenate and alkali-treated brain homogenate in conjunction with purified goat-brain cathepsins B and L at various pHs : (O) treated at pH 7.1 and then autolysed by adding enzymes, (□) homogenate and (●) treated at pH 7.1.

Experimental

Goat brain was purchased freshly from the local slaughter house. Bovine serum albumin (BSA), *p*-chloromercuribenzoic acid (PCMB) and dithioerythritol (DTE) (all Sigma), pepstatin and leupeptin were purchased from the Peptide Institute, Japan, phenol reagent, EDTA, trichloroacetic acid (TCA), diethylbarbituric acid disodium salt and other chemicals (all Glaxo) were used. Absorption was measured on Shimadzu-140 (200—1000 nm) and EC (350—900 nm) spectrophotometers. A Remi C-24 refrigerated centrifuge with a fixed angle head rotor (8×25 ml) was used for centrifugation in subcellular fractionation experiments.

Preparation of brain homogenate: The fresh goat brain tissue was washed thoroughly with cold 0.9% NaCl solution to remove blood and was cut into small pieces. It was then homogenised (3×15 s) in 10 volumes of 0.1*M* sodium acetate buffer (pH 5.5) containing 0.2*M* NaCl, EDTA (1 m*M*) and 0.1% Triton X-100 in a mixer-cum-blender. The resulting 10% (w/v) homogenate was stored at 4°.

Assay for proteolytic activity: The proteolytic activity was examined through 'autolysis' by allowing

the various protein substrates present in the goat brain homogenate to be digested by the constituent tissue proteases. The autolysis was carried out over the pH range 2.0—10.0 at 37° using 0.02875*M* universal buffer consisting of boric acid, citric acid, 5,5′-diethylbarbituric acid and NaH_2PO_4 as the incubation medium. Enzyme homogenate (1.0 ml) was mixed with equal volume of universal buffer (preadjusted to a particular pH) and the resulting 5% enzyme homogenate was incubated at 37° for 3 h. The reaction was stopped by adding TCA (3.0 ml). After centrifugation, the precipitated material was removed by filtration and the protease activity was estimated as TCA-soluble peptides either by measuring absorbance at 280 nm or by quantitating the blue colour at 660 nm, developed by using phenol reagent as described earlier[19]. Both the methods gave similar results ; the latter was, however, more sensitive.

Subcellular fractionation by differential centrifugation : The goat brain was homogenised in 0.25*M* sucrose solution in the Potter-Elvehjem-type glass homogeniser equipped with a motor-driven teflon-pestle rotating at moderately high speeds. A single pass against the pestle usually sufficed to disperse the tissue even without prior mincing while more than one pass against the pestle caused rupture of the lysosomal membrane. The method was standardised for getting reproducible results. The resulting homogenate was centrifuged at 800×g for 10 min and the pellet consisting of nuclei, unbroken cells, erythrocytes, vascular fragments and other tissue debris was designated as 'nuclear fraction'. The supernatant and the washings of the above fraction were combined and centrifuged at 16000×g for 30 min. The resulting pellet was washed with 0.25*M* sucrose and was called 'mitochondrial-lysosomal fraction'. The supernatant combined with the washings of the above fraction was designated as 'soluble fraction'. The latent hydrolase activities in these fractions were released by homogenising them separately in the presence of Triton X-100 (final concentration, 0.2%).

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