PROTEOLYTIC ACTIVITY OF RABBIT BRAIN TISSUE AT VARIOUS pH VALUES

AMARJIT KAUR AND HARI SINGH
Department of Chemistry, Kurukshetra University, Kurukshetra 132 119, India.

ABSTRACT

Proteolytic activity in the rabbit brain homogenate was determined by using haemoglobin and casein as substrates. It is found that the brain tissue contains proteases active over the entire pH range from pH 2.5–10.5. Three main peaks of activity were at pH 3.5–4, 4.5–5.5 and 8–9.5. By using pepstatin, a specific inhibitor of cathepsin D, it was shown that while the activity of the first peak was mainly due to this protease, the second peak contained other proteases too. The presence of cathepsin D and B has been demonstrated with haemoglobin and α-N-benzoyl-D, L-arginine-β-naphthylamide as substrates. The use of leupeptin, a specific inhibitor of cathepsin B, has shown that all the BANA-hydrolyzing activity is not accounted for, by this protease; other proteases like BANA-hydrolase and cathepsin L may be involved.

INTRODUCTION

THE hypothesis that small bio-active peptides present in the central nervous system act as neurotransmitters and possess hormone regulatory properties has generated tremendous interest in the mechanism of their formation and inactivation. It is now widely believed that these processes are mediated through proteolytic enzymes. Marks[1] has reviewed in detail the involvement of peptide hydrolases in the conversion and the inactivation of some neuropeptides.

A literature survey revealed that most of the studies in brain proteases focussed their emphasis only on two types of proteolytic enzymes; those having pH optimum in neutral and alkaline range and those which are maximally active at acidic pH. The peptide hydrolases purified from brain tissue which act at neutral and basic pH include two endopeptidases[2] and some exopeptidases[1,3]. The studies carried out with regard to acid-acting brain proteases[1,4-6] almost invariably conclude that this activity was due to cathepsin D (EC 3.4.23.5). Oja and Oja[7], however, studied the age-related changes in the proteinases of developing rat brain with reference to pH but did not attempt to identify them. We report here that the rabbit brain contains proteases active over almost the entire pH range. By using specific inhibitors like pepstatin and leupeptin, the presence of cathepsins D, B (EC 3.4.22.1), L (EC 3.4.22.-) and BANA-hydrolase is suggested.

MATERIALS AND METHODS

Rabbit brain acetone powder was supplied by Pel-Freeze Biological Inc., Rogers, Arkansas (USA). Bovine serum albumin (BSA), *p*-chloromercuribenzoic acid (PCMB), α-N-benzoyl-D, L-arginine-β-naphthylamide (BANA), dithieorythritol (DTE), Fast Garnet GBC dye and haemoglobin were purchased from Sigma Chemical Co., USA. Leupeptin and pepstatin were from Peptide Institute Inc., Osaka (Japan). Absorption in the UV/visible region was measured on Beckman DU-2 and EC Spectrophotometer (350-950 nm range).

Preparation of enzyme homogenate

The rabbit brain acetone powder (10 g) was suspended in 100 ml of 50 mM sodium acetate buffer pH 5.5, containing 0.2 M NaCl. After stirring at 4° C for 1 hr, it was homogenized thrice for a period of 15 sec each time in an Osterizer blender and was further stirred for 1 hr at 4° C. Protein was estimated according to the method of Lowry *et al.*[8]

Assay for proteolytic activity

The protease activity of the brain homogenate at various pH values were estimated with haemoglobin (pH 2–6) and casein (pH 6–11) as substrates. Haemoglobin (4%) was dialyzed against several changes of ion-free distilled water for 48 hr. Casein was used (4% solution) in 50 mM sodium phosphate buffer, pH 7.5. The assay mixtures (2 ml) prepared as detailed below were incubated for 3 hr at 40° C after which the reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid (TCA). The protease activity given by TCA-soluble peptides was estimated either by measuring absorption at 280 nm or colorimetrically as described earlier[9].

Enzyme assays

The activities of cathepsins D and B were assayed with denatured haemoglobin and BANA as substrates[9]. Cathepsin D activity was expressed as μ mol of tyrosine equivalents liberated/hr/ml enzyme homogenate. For this purpose, a calibration curve was prepared using L-tyrosine as standard.

Results and Discussion

Proteolytic activity profile at different pHs

As large amounts of protein present in the enzyme homogenate could alter the pH of the final incubation mixture, some preliminary experiments were conducted to standardize the procedure. Accordingly, equal volumes of the appropriate buffers (0.2 M) and substrates (4%) were mixed and by adjusting the pH of the resulting 2% substrates in 0.1 M buffers with 1N HCl or 1N NaOH, 12 substrate stock solutions having pH values of 1.00, 1.51, 2.01, 3.03, 3.34, 4.22, 5.09, 6.00, 7.99, 9.90, 10.49 and 10.98 were obtained. When 1 ml out of each of these substrate stocks was mixed with equal volume of the enzyme homogenate, the pH values of the resulting 2 ml incubation mixtures were found to be 2.70, 3.45, 3.77, 3.98, 4.51, 4.77, 5.30, 5.88, 7.38, 7.88, 9.52 and 10.38. These samples were prepared in quadruplicate at each pH and the assays were done in triplicate and the fourth sample was used to measure the final pH of the assay mixture. After 3 hr incubation at 40° C, the TCA-soluble peptides in the filtrate were estimated as described earlier. Five experiments done at different times and with different enzyme preparations gave identical results with regard to the positions of the peaks. The results of one typical experiment are represented in figure 1. It is evident that the rabbit brain homogenate contains proteolytic activity spread over the entire pH range (2.5–10.5). Three main peaks of activity are in the regions of pH 3.5–4, 4.5–5.5 and 8–9.5. Similar results were obtained when the activity was expressed in terms of OD_{280} units.

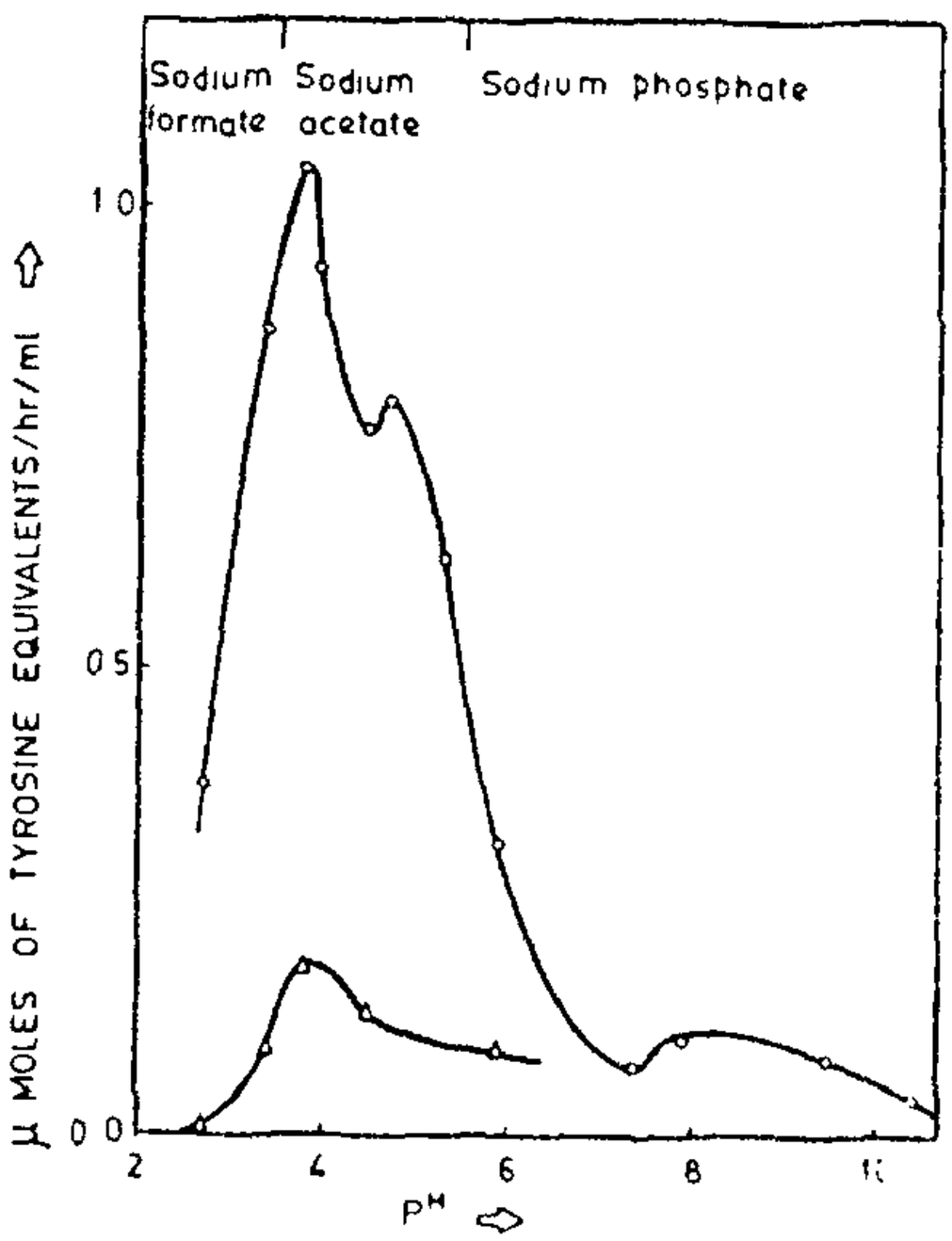

Figure 1. Proteolytic activity of rabbit brain tissue at different pH values. Haemoglobin was the substrate upto pH 6 and casein was used as substrate between pH 6–10.5. Assays were done in triplicate at 40° C. Both substrate and enzyme blanks were included. The reaction was stopped by the addition of TCA and the digestion products in the filtrate were estimated colorimetrically by Folin's reagent. The results are expressed as μ mol of tyrosine equivalents released/hr/ml homogenate. -o-o-, activity without pepstatin, and -△-△-, activity with pepstatin.

Inhibition of protease activity by pepstatin

Since the presence of acid protease cathepsin D has been reported in brain tissue by many workers[1,4-6,10,11], it was worthwhile to know whether all the protease activity in the acid pH range was accounted for by cathepsin D. Pepstatin, acetyl/isovaleryl-L-valyl-L-valyl-AHMHA-L-alanyl-AHMHA (AHMHA is 4-amino-3-hydroxy-6-methylheptanic acid) a strong inhibitor of cathepsin D[12], was included in the assay mixture at final concentration of 0.1 mM. The results are shown in figure 1. Whereas, the inhibition of protease activity was very high (82–90%) in the pH range of 3.45 to 4.51, it was only 68% at pH 5.88. The obvious conclusions from these observations are: cathepsin D contributes towards protease activity in the first peak to a large extent, proteases other than cathepsin D are also involved in the second proteolytic activity peak. Two such pepstatin-insensitive proteases from other tissues[12,13] which degrade protein substrates maximally between pH 4–6 are cathepsin B and L; cathepsin L is particularly active against proteins and is strongly inhibited by leupeptin[12].

Activities of cathepsin D and cathepsin B

The presence of these two cathepsins was demonstrated in the brain homogenate using haemoglobin at pH 3.5 and BANA at pH 6 as substrates. The total activity of cathepsin D was found to be 36.56 μmoles of tyrosine equivalents/hr and that of cathepsin B was 10.41 μmoles of β-naphthylamine released/hr in the homogenate prepared from 10 g of brain powder.

Inhibition of BANA-hydrolyzing activity by leupeptin and PCMB

Our next task was to identify the BANA-hydrolyzing enzymes. The possibility of non-specific hydrolysis of BANA by trypsin-like enzymes was dis-

counted because the BANA-hydrolyzing activity was completely abolished by 1 mM PCMB. This also implied that all the protease activity was due to thiol proteases. In the light of the results of Singh and Kalnitsky[9,14], who demonstrated that the total BANA-hydrolyzing activity of rabbit lung was due to two enzymes (cathepsin B and a new BANA-hydrolase), it was only proper to find out whether the BANA-hydrolyzing activity of brain was due entirely to cathepsin B or not. Leupeptin, acetyl-L-leucyl-L-leucyl-L-arginal, a strong inhibitor of cathepsin B[12] but not that of BANA-hydrolase[9] was included in the assay mixtures. At 0.5 μM concentration, sufficient to inhibit completely the activity of purified rabbit lung cathepsin B[9], the inhibition of BANA-hydrolyzing activity in the brain homogenate was only about 80%. Thus, the presence of other BANA-hydrolases insensitive to leupeptin is definitely indicated.

The analysis of the results on the proteolytic activity of rabbit brain homogenate afford the following conclusions: the first peak of protease activity between pH 3.5–4 is mainly due to cathepsin D and/or other related carboxyl endopeptidases sensitive to pepstatin. The second protease peak at 4.5–5.5 contains other proteases in addition to cathepsin D. The thiol proteases present in brain are not completely inhibited by leupeptin implying the presence of BANA-hydrolase[9,14] type enzymes. The leupeptin-sensitive enzymes are cathepsin B and L. The role of protein-hydrolyzing leupeptin-sensitive cathepsin L can only be speculative because its presence in brain has not been reported yet. The work in this direction is in progress. The enzymes in the protease activity peak between pH 8.0–9.5 represent the neutral and alkaline proteases and the study of their characteristics is underway.

ACKNOWLEDGEMENTS

The authors thank Prof. George Kalnitsky and Dr. Ranjit Chatterjee, Department of Biochemistry, University of Iowa, Iowa City, Iowa (USA) for the generous gift of substrates and inhibitors. Thanks are due to Prof. H. K. Pujari for his keen interest in the work and to Dr. D. Mukherji for the use of EC-spectrophotometer. We are grateful to the Head of the Chemistry Department for providing facilities.

1. Marks, N., in *Peptides in Neurobiology*, (ed.) H. Gainer, Plenum Press, New York, 1977, p. 221.
2. Wilk, S. and Orlowski, M., *J. Neurochem.*, 1980, **35**, 1172; Orlowski, M., Wilk, E., Pearce, S. and Wilk S., *J. Neurochem*, 1979, **33**, 461.
3. Hersh, L. B. and McKelvy, J. F., *J. Neurochem.*, 1981m **36**, 171.
4. Whitaker, J. N., *J. Neurochem.*, 1980, **34**, 284.
5. Oja, S. S. and Heinaemaeki, A.A., *Chem. Abstr.*, 1981, **94**, 12082g.
6. Marks, N., Benuck, M. and Hashim, G. A., *J. Neurosci. Res.*, 1980, **5**, 217.
7. Oja, S. S. and Oja, H., *J. Neurochem.*, 1970, **17**, 901.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, **193**, 265.
9. Singh, H. and Kalnitsky, G., *J. Biol. Chem.*, 1978, **253**, 4319.
10. Berezin, V. A., Reva, A. D., Shmatchenko, N. A. and Korobov, V. I., *Chem. Abstr.*, 1979, **91**, 86216p.
11. Organisyan, A. I., Akopyan, T. N., Gevorkyan, G. A. and Galoyan, A. A., *Chem. Abstr.*, 1980, **92**, 195512y.
12. Barrett, A. J., in *Proteinases in mamallian cells and tissues*, (ed.) A. J. Barrett, North-Holland Publishing Co., Amsterdam, 1977, p. 181.
13. Singh, H., Tsungmin, K. and Kalnitsky, G., in *Protein turnover and lysosome function*, (eds) H. Segal and D. J. Doyle, Academic press, New York, 1978, p. 315.
14. Singh, H. and Kalnitsky, G., *J. Biol. Chem.*, 1980, **225**, 369.

ANNOUNCEMENT

THE INTERNATIONAL ASSOCIATION FOR THE SCIENTIFIC STUDY OF MENTAL DEFICIENCY

The International Association for the Scientific Study of Mental Deficiency presented the Distinguished Achievement award to Dr. R. M. Varma famous neuro-surgeon for significant contributions to the improvement of services for mentally deficient persons throughout the world. The award was presented on August 25th at the VI Triennial Congress, Toronto, Canada.