KINETIC AND MECHANISTIC STUDIES ON CARBOXYPEPTIDASE A (EC 3.4.12.2) FROM GOAT PANCREAS

BY

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DEDICATED

TO

MY PARENTS

C E R T I F I C A T E

This is to certify that the thesis entitled, "Kinetic and Mechanistic Studies on Carboxypeptidase A (EC 3.4.12.2) from Goat Pancreas" being submitted by Kamlesh K. Gupta to the Indian Institute of Technology, Delhi for the award of the degree of 'Doctor of Philosophy', is a record of bonafide research work carried out by her. Kamlesh K. Gupta has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge, has reached the requisite standard.

The results contained in this thesis have not been submitted in part or full, to any other University or Institute for the award of any degree or diploma.

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A C K N O W L E D G E M E N T S

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ABSTRACT

The molecular weight of goat carboxypeptidase A was found to be approximately 34,600 by Gel-filtration and SDS-method. It was found to be a zinc-metalloprotein both by chemical and emission spectroscopy methods, having one atom of zinc per protein molecule. Removal of zinc either by dialysis at pH 6.0 or below and by the use of chelating agents at neutral pH, yielded an inactive apocarboxypeptidase A. The essentiality of this metal ion for enzymic activity and possibility of substitution of the native metal by other metal ions with retention of peptidase or esterase activity, using dialysis and preincubation procedures, have been established. Substitution of zinc by other metal ions effects both K_m and V_m values and, therefore, it has been concluded that metal ion is involved in binding as well hydrolysis of the substrate.

Chemical modification studies with reagents, specific for the side chain of amino acid residues most likely to be present at the active site of the enzyme, indicated the involvement of arginine, tyrosine, glutamic/aspartic acid and histidine at the active site. Optimal conditions have been established for the reaction specific for each side chain of these amino acid residues and loss of the enzyme activity has been correlated with the modification of side chain of amino acid residue in every case.

Goat carboxypeptidase A showed optimal activity at 50°C while studies on thermal denaturation of the enzyme showed that it starts losing activity above 35°C. So, thermal stability of the enzyme was studied in the presence of substrate or one of its products which indicated that the enzyme is stabilised by only L-phenylalanine, one of the reaction products. Effect of order of addition of products and substrate in the incubation mixture suggested that the slowest step during the hydrolysis of hippuryl-L-phenylalanine is the release of L-phenylalanine. The slow release of L-phenylalanine could be explained due to hydrogen bond formation between amino group of product: L-phenylalanine and active site glutamic acid and tyrosine. Hydrogen bonding was further confirmed by pmr studies. The order of release of products was also confirmed by kinetic studies, which showed that while the presence of hippuric acid in the medium has no effect on the rate of substrate hydrolysis, L-phenylalanine inhibits the reaction competitively.

On the basis of involvement of arginine, tyrosine, glutamic acid and histidine at the active centre, essentiality of the zinc ion, thermal stability, pmr and kinetic studies a new mechanism has been proposed. Its basic features are similar to that proposed by Lipscomb[72,76] for carboxypeptidase A mediated hydrolysis of acyldipeptides but differs from it in the order of release of the reaction products. We propose that hippuric acid is released first followed by L-phenylalanine. Moreover, release of L-phenylalanine is the rate determining step.

This slow release of this product is due to electrostatic interactions between amino group of L-phenylalanine and active site amino acid residues. Glutamic acid acts as a base rather than a nucleophile. This mechanism can explain the lack of transpeptidase activity, lack of effect of solvent on reaction rates and failure to pick up label when the reaction has been performed in the presence of $^{14}CH_3OH$, because no acid anhydride intermediate is visualised in our proposed mechanism as is the case with Lipscomb's proposal and the slowest step is release of L-phenylalanine.

C O N T E N T S

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