Crystalline papain

VI. EXTENSIVE STEPWISE HYDROLYSIS OF MERCURIPAPAIN BY LEUCINE AMINOPEPTIDASE WITHOUT LOSS OF PROTEOLYTIC ACTIVITY*

Highly purified leucine aminopeptidase (LAP) from swine kidney[1] can liberate amino acids sequentially from the N-terminal end of synthetic peptides, oxidized A and B chains of insulin, and a number of proteins[2,3]. It has already been reported that with low concentrations of LAP, 19 residues are liberated from enzymically inactive mercuripapain (MP)[4] in essentially stoichiometric amount in 24 hours, with no loss in activity of the degraded papain after removal of mercury[2,3]. The molar ratios of the amino acids liberated from MP differ markedly from the molar ratios of complete hydrolysates of intact MP. Instead of the original N-terminal residue of ileu identified as the dinitrophenyl (DNP) derivative[5], the degraded residual MP when treated with fluorodinitrobenzene (FDNB)[6] gave no DNP-ileu but mostly DNP-arg, some DNP-phe and traces of DNP-ala, DNP-asp and DNP-gly.

It was thought at first that LAP could remove only 19 residues from the N-terminal end of MP. It has now been found that more extensive degradation of MP can be accomplished when a higher ratio of LAP to MP is employed. Fig. 1 shows the extent of hydrolysis of MP in 24 hours with different ratios of LAP to MP, as measured by a colorimetric ninhydrin method[6]. Since the increased ninhydrin color is due entirely to free amino acids and not to peptides, the number of residues liberated is assessed on the basis of the color yield given by leucine. Since leucine gives maximal color yield with ninhydrin and a few amino acids give lower color yields, *e.g.*, proline, the number of residues liberated may be assumed to be minimal. LAP is thus able to proceed stepwise through 120 of the 180 residues present in the single peptide chain of MP**. Control samples of MP without LAP or of LAP without MP show no change in ninhydrin color under the same conditions.

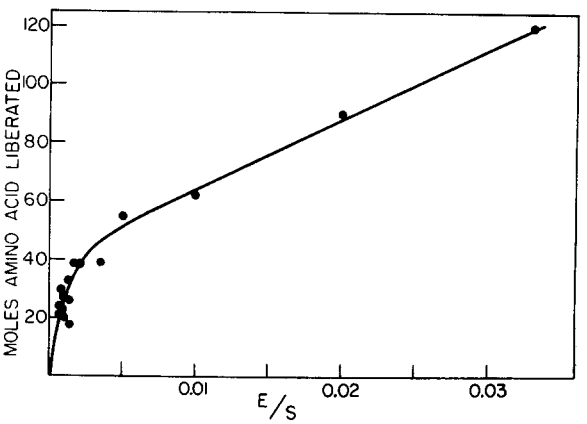

Fig. 1. Amount of hydrolysis given as moles of amino acid liberated (from ninhydrin color) per mole of mercuripapain. Molar ratio of enzyme (LAP) to substrate (MP) given as E/S on the basis that the molecular weight of LAP is 15 times that of MP. Extent of hydrolysis was determined after incubation at 40° C for 24 hours at pH 8.5 ± 0.1 in the presence of 0.001 *M* $MgCl_2$ and 0.005 *M* Veronal or Tris buffer. Highly purified preparations of LAP[1] were used after treatment with di*iso*propyl fluorophosphate (DFP).

The striking feature is that after removal of mercury all the degraded preparations of MP exhibit the same *molar* activity towards benzoyl-L-arginin-amide (BAA) as do undigested, control samples of MP kept under the same conditions***. Tests with MP preparations (after removal of the mercury) which had been degraded by 19 or 39 residues show no change in absolute or relative activity toward other synthetic substrates for the enzyme, *e.g.*, carbobenzoxy-L-glutamic acid diamide, hippuryl amide and carbobenzoxy-L-leucinamide. More extensively hydrolysed preparations of MP have been tested thus far only with BAA as substrate.

Further evidence that MP is extensively hydrolysed was obtained by the DNP method. After an apparent removal of 39 residues by LAP and separation from the liberated free amino acids, the residual MP was treated with FDNB, the DNP-protein hydrolysed and the DNP

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** Papain is assumed to consist of a single peptide chain because of the presence of only one α-amino group[4]. The molecule does not appear to contain disulfide bridges as judged by the agreement between the total number of sulfhydryl groups and the recovery of cysteic acid from preparations oxidized with performic acid (E. L. SMITH, B. J. FINKLE AND A. STOCKELL, Discussions Faraday Soc., August 1955, in press).

*** Assays for papain activity were performed as previously described[4]. LAP has no activity towards BAA[1] and is completely inactivated under the conditions used for the papain assay.

amino acids isolated by the usual methods. No DNP-ileu, the original N-terminal residue, or DNP-arg, the predominant end group found after removal of 19 residues, could be detected. Instead, large amounts of DNP-val, DNP-asp and smaller amounts of DNP-ser, DNP-ala and DNP-glu were found. A mixture of end groups is to be expected, since the enzymic degradation by LAP progresses stepwise and independently in each molecule of MP.

From these experiments we can conclude that the active site of papain is independent of and distant from the N-terminal end of the protein molecule. Just how large the active site must be has still not been demonstrated but the removal of approximately 120 ± 10 residues (Fig. 1) of the original 180 in the protein[8] suggests that even more extensive degradation to an active residue may be possible. Quantitative analyses are now in progress to determine precisely the amino acids liberated and the composition of the undegraded part of the papain molecule at different stages of degradation. The above experiments demonstrate the great utility of an enzyme such as LAP in the degradation of proteins and polypeptides, which can liberate all amino acids normally present in proteins only by hydrolysis at a bond adjacent to a free α-amino group. However, it should be emphasized that not all native proteins are readily susceptible to the action of LAP, *e.g.*, human serum albumin is not attacked by LAP but is hydrolysed after oxidation with performic acid. In addition to its use as a reagent for studying the relationship between protein structure and biological activity, LAP is being used in our laboratory for the determination of the sequence in polypeptides and proteins by stepwise methods analogous to those in which carboxypeptidase has been used[9,10].

Limited proteolysis of a number of enzymes without loss of activity has already been accomplished in other cases. For the autolysis of pepsin[11] and the action of subtilisin on ribonuclease[12], the sites of action of the proteinases are unknown. With carboxypeptidase, removal of one or a few residues from the C-terminal end of lysozyme[13] and ribonuclease[14] does not influence the enzyme activity. The extensive degradation of some enzymes, *e.g.*, papain and pepsin, taken in conjunction with evidence that rupture of many non-covalent bonds does not influence the activity of ribonuclease[15], indicates that intact protein structure is not essential for enzymic activity. Such studies offer further support for the concept of an "active site" which does not require the participation of more than a part of the entire protein structure of an enzyme[16].

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