The Use of Thermolysin in Amino Acid Sequence Determination

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(Received 22 May 1968)

Thermolysin is a protease from the thermophilic bacterium *Bacillus thermoproteolyticus* Rokko (Endo, 1962). The enzyme has a specificity that is quite different from that from any of the proteases previously available for sequence investigations, as has been shown by studies on synthetic peptide substrates (Ohta & Ogura, 1965) and on proteins of known structure (Matsubara, Singer, Sasaki & Jukes, 1965; Matsubara, Sasaki, Singer & Jukes, 1966). These studies have shown that normally thermolysin will hydrolyse the peptide bonds on the *N*-terminal side of hydrophobic residues.

The present investigation was undertaken to see how useful this enzyme would be for investigation of amino acid sequences, and to clarify points about its specificity. *Pseudomonas* azurin, a small protein of known structure (Ambler & Brown, 1967), was used as substrate, the products of digestion under different conditions were fractionated and an estimate of the thermolysin-sensitive bonds in the molecule was made. These results were compared with the known effects of trypsin, chymotrypsin, pepsin and subtilisin B on the molecule. The specificity of thermolysin was also examined with peptides of known sequence derived from *Pseudomonas* azurin.

Methods and materials. Thermolysin was obtained from Chugai Boyaki Co. Ltd., P.O. Box Higashi no. 106, Osaka, Japan. *Pseudomonas* apoazurin and azurin peptides were prepared as described by Ambler & Brown (1967). Except where otherwise mentioned, the methods used were those of Ambler (1963) and Ambler & Brown (1967).

The conditions used for digestion were very similar to those that have been used for digestions with trypsin, chymotrypsin and subtilisin B (Ambler, 1963). Thermolysin (0.5 mg./ml.) was dissolved in ammonium acetate (0.2N with respect to acetic acid), pH 8.5, containing 5 mM-CaCl₂. Protein (20 mg./ml.) or peptide (1–0.1 μ mole/ml.) was dissolved in the same buffer, and after addition of enzyme incubated at 37° or 57° for $\frac{1}{4}$ –7 hr. The enzyme/substrate ratios used were from 1:20 to 1:100 (by wt.) for protein, and from 0.02–0.5 mg./ μ mole for peptides. After digestion the mixtures were evaporated to dryness in a vacuum desiccator.

The mixtures were fractionated by gel filtration (whole protein digests only), high-voltage paper

electrophoresis and paper chromatography, and the isolated peptides were identified by qualitative analysis for amino acid composition and *N*-terminal group. In a few cases the identity of a peptide was confirmed by *C*-terminal analysis (with carboxypeptidases) or by quantitative amino acid analysis.

Action of thermolysin on azurin. Fig. 1 shows the peptides isolated from a thermolysin digest of apoazurin (5 μ moles, 57°, 1 $\frac{1}{2}$ hr., enzyme/substrate ratio 1:50 by wt.). Also shown are those peptides isolated from a digest of performic-acid oxidized apoazurin (2.5 μ moles, 37°, 3 hr., enzyme/substrate ratio 1:80 by wt.) that were not detected in the digest of unoxidized protein.

Small-scale experiments showed no qualitative difference between digests at 37° and 57°. Under milder conditions of digestion (time, temperature or enzyme/substrate ratio) two peptides (residues 1–14 and 111–119) were produced in greater quantity, whereas after more vigorous digestion two further peptides (residues 81–83 and 117–119) had increased in amount. In general the differences in peptide pattern between digestions at the extremes of the ranges of conditions were very small.

The effect of the proximity of free α -amino and carboxyl groups on the action of thermolysin was investigated with azurin peptides that contained bonds shown to be sensitive to the enzyme when in the intact protein. The tripeptides Asp-Val-Ser (98–100), Thr-Val-Asn (30–32), Mes*-Phe-Phe (109–111), Mes-Gln-Phe (13–15), Asn-Val-Mes (42–44) and Trp-Val-Leu (48–50) were not split, nor were the larger peptides Leu-Ile-Gly-Ser (86–89) and Asp-Val-Ser-Lys-Leu (98–102). Peptides were split by thermolysin when they contained bonds that were both susceptible in the whole protein and not adjacent to either end. Thus Val-Ile-Ala-His-Thr-Lys (80–85) was cloven at His-Thr, Ser-His-Pro-Gly-Asn-Leu-Pro-Lys-Asn-Val-Mes (34–44) at Asn-Val (but not at Asn-Leu), and peptides containing the sequence 113–121 were split at Gly-His and Ala-Leu, but not at Thr-Phe.

Specificity of thermolysin. The results described above confirm that thermolysin rapidly hydrolyses peptide bonds on the *N*-terminal side of valine, leucine, isoleucine and phenylalanine residues.

* Abbreviation (in amino acid sequences): Mes, methionine sulphone.

Fig. 1. Peptides formed by the action of thermolysin on *Pseudomonas fluorescens* azurin. ◄—►, Peptides isolated in high yield; ◄----►, peptides isolated in moderate yield; ◄.....►, peptides isolated in low yield; ◄--►, peptides isolated only from digest of performic acid-oxidized azurin. The peptides were isolated by gel filtration followed by high-voltage paper electrophoresis and paper chromatography, and identified by qualitative amino acid analysis and *N*-terminal group determination. The amino acid compositions of peptides labelled * were determined quantitatively.

Bonds on the *N*-terminal side of methionine residues are also cloven, as would be predicted from the hydrophobic nature of the side chain, but if the substrate is oxidized bonds on the *N*-terminal side of methionine sulphone residues are not affected. Most bonds on the *N*-terminal side of tyrosine residues, and some (but not all) on the *N*-terminal side of alanine residues are split. A small proportion of bonds on the *N*-terminal side of asparagine, threonine, histidine and glycine residues are susceptible, but not enough cases have yet been encountered for it to be apparent what distinguishes these sites. The bonds on the *N*-terminal side of the cystine and cysteine residues in azurin show a slight susceptibility to hydrolysis, but no such effect has been found in insulin.

Thermolysin does not appear to be able to hydrolyse peptide bonds on the *N*-terminal side of a hydrophobic residue if the next but one residue is proline (e.g. bonds 38–39 and 113–114). In azurin there is no case of a proline residue on the *N*-terminal side of a hydrophobic residue, but thermolysin splits Pro-Ile and Pro-Val bonds in staphylococcal penicillinase (R. P. Ambler, unpublished work).

Our results with natural tri-, tetra- and penta-

peptides confirm the findings of Matsubara (1966) with synthetic peptides that adjacent α -amino or carboxyl groups prevent the hydrolysis of peptide bonds by thermolysin. ω -Amino and carboxyl groups do not have a marked effect on the susceptibility of adjacent bonds.

In our experiments temperature did not have a significant effect on the specificity of thermolysin.

Thermolysin in sequence determination. In most proteins more peptide bonds will be susceptible to thermolysin than to chymotrypsin or trypsin. In azurin, 39 of the 127 peptide bonds are hydrolysed at a significant rate, compared with 14 by trypsin and 26 by chymotrypsin. Pepsin hydrolyses 32 bonds, and subtilisin B at least 52 (Ambler & Brown, 1967). Of the bonds sensitive to thermolysin, 11 are not hydrolysed by any of the other enzymes. Thermolysin digests of large proteins will generally be too complex to be worth fractionating, but the enzyme is potentially very useful for the secondary degradation of large tryptic or chymotryptic peptides, particularly those that are insoluble because of their high content of hydrophobic residues.

The enzyme has already proved extremely useful

for the primary digestion of small proteins. For instance *Desulphovibrio* cytochrome c_3 contains only 12 bonds susceptible to chymotrypsin, and most of the chymotryptic peptides are large and difficult to purify, whereas thermolysin hydrolyses 18 bonds, and the peptides are easy to purify, and in conjunction with the tryptic peptides allow the sequence of the protein to be established (Ambler, 1968).

Thermolysin digests contain many peptides that have *N*-terminal valine or isoleucine, and so are likely to have low colour yields with ninhydrin. Great care must be taken to ensure that such peptides are not lost, particularly if paper methods are used for purification.

R. J. M. holds a Medical Research Council Scholarship for Training in Research Methods.

Ambler, R. P. (1963). *Biochem. J.* **89**, 349.

Ambler, R. P. (1968). *Biochem. J.* (in the Press).

Ambler, R. P. & Brown, L. H. (1967). *Biochem. J.* **104**, 794.

Endo, S. (1962). *J. Ferment. Tech.* **40**, 346.

Matsubara, H. (1966). *Biochem. biophys. Res. Commun.* **24**, 427.

Matsubara, H., Sasaki, R., Singer, A. & Jukes, T. H. (1966). *Arch. Biochem. Biophys.* **115**, 324.

Matsubara, H., Singer, A., Sasaki, R. & Jukes, T. H. (1965). *Biochem. biophys. Res. Commun.* **21**, 242.

Ohta, Y. & Ogura, Y. (1965). *J. Biochem., Tokyo*, **58**, 607.