The proteinase-catalysed synthesis of peptide hydrazides

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1. We report the trypsin-catalysed conversion, in high yield, of peptides to peptide hydrazides, t-butyloxycarbonylhydrazides and phenylhydrazides. The substitution is at the α -carboxy group. 2. We discuss the relative merits of carrying out the conversion either simultaneously with tryptic cleavage of the parent protein or after such cleavage. 3. We report analogous results with chymotrypsin, elastase and subtilisin. 4. We propose the use of such products in protein semi-synthesis and in the preparation of specific proteinase inhibitors.

Peptide α -hydrazides, whether substituted or not in the hydrazo function, are useful intermediates in the synthesis or semi-synthesis of longer polypeptide chains (Bodanszky *et al.*, 1976; Offord, 1980). The α -hydrazide is the required starting material for the azide method for the formation of peptide bonds (Curtius, 1902, 1904); the peptide hydrazide is converted into the azide by nitrite ion, and the azide reacts in its turn with the α -NH₂ group of the molecule to which the peptide is to be coupled. The amino groups of the peptide hydrazide should be reversibly protected both against the possible action of the nitrite during the conversion into azide and against reaction with azide once it is formed, but an attempt to protect such amino groups in the peptide hydrazide would normally lead to protection of the hydrazide function as well. It is therefore sometimes advantageous to form a substituted hydrazide and then protect the amino groups of the peptide with a group having different properties to that used to protect the hydrazide function itself. These reasons led Schwyzer *et al.* (1960) to the proposed use of t-butyloxycarbonylhydrazides of the α -carboxy group. Phenyl substitution of the hydrazide function is also of potential use, since the protection can be removed at will by oxidation (Waldschmidt-Leitz & Kühn, 1951). Peptide α -phenylhydrazides have also been used directly in the formation of peptide bonds, since oxidation by *N*-bromosuccinimide produces a

phenyldi-imide capable of direct reaction with an amino group (Milne & Kilday, 1965).

Chemical methods exist for the preparation of such compounds, for example the hydrazinolysis of peptide α -carboxy esters (Bodanszky *et al.*, 1976), but the success of Homandberg & Laskowski (1979) in developing general methods for employing reverse proteolysis for the formation of peptide bonds for other than model compounds has raised the question of whether one might similarly use enzymes for the direct preparation of the hydrazide. There are many cases in which the component to which the peptide is to be coupled would be damaged by direct exposure to the proteinase and an intermediate of the hydrazide type is therefore required. Canova-Davis & Carpenter (1980) obtained by such means a sample of insulin in which the last eight residues of the B-chain had been removed and replaced, with an efficiency of 30%, by a hydrazo group.

While the present work was in progress, Yagisawa (1981) reported the formation of esters, hydrazides, Boc-hydrazides and Cbz-hydrazides of model peptides by the reverse reaction of trypsin. The present paper confirms the applicability of this principle to longer, natural peptides under somewhat different conditions and extends it to the preparation of peptide phenylhydrazides. Scheme 1 illustrates the type of reaction carried out in the present work. In addition we find that the hydrazide derivatives can be formed concurrently with cleavage of the peptide from its parent, as well as by action of the enzyme on the purified peptide. Efficiencies are in most cases close to 100%. Also, we find chymotrypsin, elastase and subtilisin to be equally applicable in this method.

Abbreviations used: Boc, t-butyloxycarbonyl; Cbz, benzyloxycarbonyl.

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Scheme 1. *Typical synthesis of a peptide hydrazide*
 R = H (hydrazine), C₆H₅ (phenylhydrazine), (CH₃)₃C—O—CO— (t-butyloxycarbonylhydrazine)

Materials and methods

Hydrazine hydrate and phenylhydrazine were fresh samples of BDH laboratory-reagent grade (BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.). t-Butyl carbazate ('97%') was from Aldrich (Aldrich-Europe, Janssen Pharmaceutica N.V., B-2340, Barse, Belgium). These reagents were used without further purification. Hydrazine acetate was prepared by adding hydrazine hydrate (5 ml) to acetic acid (5.7 ml) with cooling and stirring. After the addition the mixture set to a crystalline cake, which gave satisfactory results without any further treatment.

All other materials and general methods were as described by Webster & Offord (1977), Rees & Offord (1976a,b) or Gonzales & Offord (1971). The activation peptide from bovine cationic trypsinogen was isolated from trypsin-treated trypsinogen by ion-exchange chromatography on a column (8.5 cm × 1.5 cm) of DEAE-Sephadex A-25 eluted at 40 ml/h with (a) 80 ml of the equilibration buffer (100 mM-ammonium formate, pH 7.0) followed by (b) a linear gradient of 20–60 mM-ammonium formate/formic acid, pH 3.5 (200 ml). The effluent containing the hexapeptide emerged between 81 and 108 ml after the start of the gradient. It was freeze-dried, and the resulting material fractionated by high-voltage paper electrophoresis at pH 6.5.

Performic acid-oxidized lysozyme was prepared by adding 10 ml of performic acid to 250 mg of lysozyme. The performic acid was made by slowly adding, with mixing, 9.5 ml of 98–100% (w/v) formic acid (pre-cooled in ice for 10 min) to 0.5 ml of 30% (v/v) H₂O₂, which had also been pre-cooled. The

protein solution was left 30 min at 20°C and was then exhaustively dialysed against distilled water. The contents of the sac (pH 5.0) were adjusted to pH 8.0 by adding 1.0 ml of 1 M-NH₃, and then freeze-dried.

The conversion into hexapeptide hydrazide was carried out in the following solution, at an apparent pH of 8: peptide (3.2 mM); butane-1,4-diol, 52% (v/v); hydrazine acetate, 1.7 M; CaCl₂, 43 mM; trypsin, 5.4 mg/ml. This was achieved in practice as follows. To a freeze-dried sample of peptide (0.45 μmol) was added 72 μl of butane-1,4-diol followed by 3 μl of distilled water. To this was added 26.4 mg of hydrazine acetate, prepared as above. To this mixture was then added 3 μl of 2 M-CaCl₂ and 12 μl of 1 M-NH₃ (which brought the pH, as estimated by an external indicator, to 8). To this solution was added 30 μl of aqueous trypsin (25 mg/ml). The reaction mixture was then incubated at 37°C. When it was desired to use different amounts of trypsin, the appropriate volume of 25 mg/ml solution was used, and the difference in the amount of water added with the trypsin was compensated for by the appropriate change in the volume of water added with butane-1,4-diol.

The conversion into hexapeptide Boc-hydrazide was carried out as described above, except that the hydrazine acetate was replaced by 31.7 mg of solid t-butyl carbazate (t-butyloxycarbonylhydrazine), followed by 6 μl of 0.75% (v/v) acetic acid. The use of an external indicator showed that the pH was 8 without the need for further adjustment.

The conversion into hexapeptide phenylhydrazide was carried out in the same way as that of the Boc-hydrazide except that the t-butyl carbazate was replaced by 24 μl of phenylhydrazine, and that

12 μ l of 12.5% (v/v) acetic acid was then added. The pH was 6.5 as measured with an external indicator.

The reaction mixtures were incubated at 37°C. The reaction was stopped by the addition of 8 μ l of formic acid (98–100%, w/v). The pH as observed with an external indicator was approx. 2. The mixture was then diluted with an equal volume of 7% (v/v) formic acid, and applied to a column (42.6 cm $\times$ 0.6 cm) of Sephadex G-25, also in 7% formic acid.

The hexapeptide and its derivatives were located in the column effluent, and a visual estimate was made of their relative amounts by high-voltage paper electrophoresis at pH 6.5 of freeze-dried fractions. The peptide had an electrophoretic mobility (m) of -0.83 [measured as described by Offord (1966)] and for the hydrazide $m = -0.61$, both relative to $m_{\text{aspartic acid}} = -1.0$. The electrophoresis showed that the peptide and its hydrazide and Boc-hydrazide derivatives emerged at 0.57–0.82 column volumes, whereas the phenylhydrazide derivative emerged at 0.54–0.79 column volumes. They were entirely separated from trypsin (which was located in the effluent by u.v. absorption), but only partially separated from the hydrazines, butane-1,4-diol and salt. The peptide and peptide hydrazide fraction was freeze-dried and applied in a small volume of 100 mM-ammonium acetate, pH 7.0, to a column (8 cm $\times$ 1.3 cm) of DEAE-Sephadex A-25, previously equilibrated in the same buffer. Salt, butane-1,4-diol and hydrazine were removed with 36 ml of starting eluent. Acetic acid (2%, pH 2.6) was used to elute the hexapeptide hydrazides (14–22 ml) and, when present in detectable amounts, the hexapeptide itself (18 ml–54 ml).

Electrophoresis at pH 1.9 is not very suitable for the isolation of peptide hydrazides or their substituted derivatives, since there is no charge difference at this pH between a peptide and its hydrazide derivatives (R. Jones, unpublished work). Such separation as does take place depends on molecular weight differences and is seen only with the heavier substituted hydrazide groups.

Recoveries of peptide products were estimated (as described by Gonzalez & Offord, 1972) by amino-acid analyses with, in the case of less highly purified materials, the subtraction of results obtained from digests carried out without hexapeptide.

Peptide hydrazides and phenylhydrazides, but not Boc-hydrazides, could be detected on paper by the spray described by Barton *et al.* (1952).

Tryptic digests of performic acid-oxidized lysozyme in the presence of hydrazine acetate were carried out by using 14.3 mg samples of the protein exactly as for the conversion of the hexapeptide, except that all volumes were scaled up by a factor of 8.3, and that 5 μ l of 2 M-CaCl₂ solution was used. As a consequence of this latter change the final CaCl₂

concentration was 8.6 mM rather than 50 mM. A larger column (50 cm $\times$ 1.6 cm) of Sephadex G-25 was used for separation of such digests. Electrophoreses at pH 6.5 of freeze-dried samples of the effluent between 0.64 and 0.78 column volumes were inspected visually. The peptide mixture emerging from the column at this point contained, as the only component staining yellow with cadmium/ninhydrin, the peptide Thr-Pro-Gly-Ser-Arg ($m = +0.43$). The hydrazide of this peptide ($m = +0.78$, predicted value = $+0.75$) also emerged in this column fraction. For a preliminary screening of reaction conditions, the extent of hydrazinolysis of the protein was estimated by comparing the intensity of ninhydrin staining of the Thr-Pro-Gly-Ser-Arg hydrazide spot with that of the Thr-Pro-Gly-Ser-Arg spot. Both intensities were also compared with that of the free peptide obtained from a corresponding digest carried out, in the absence of hydrazine acetate, by treating 14.3 mg of performic acid-oxidized lysozyme in 0.5% (w/v) NH₄HCO₃, pH 8.0, for 90 min at a trypsin/lysozyme ratio of 1:100 (w/w). Only conditions that left essentially no visible starting peptide were studied further.

Alternatively a tryptic digest of lysozyme could be converted to the corresponding hydrazides. The digest (14.3 mg) was treated as described for the undigested protein, and the extent of hydrazide formation was estimated as described above.

The trypsin-catalysed hydrazinolysis of trypsinogen was attempted by treating 20 mg of trypsinogen in the usual way with 1 ml of 52% (v/v) butane-1,4-diol containing 1.7 M-hydrazine acetate, 43 mM-CaCl₂, 0.86 mg of trypsin/ml, pH 8.0 (adjusted with NH₃). The mixture was incubated for 1 week at 37°C.

Results

Conversion into hexapeptide hydrazide

By using the solvent system described in the Experimental section the conversion into hexapeptide hydrazide was essentially complete after a 1 week's incubation with 1.08 mg of trypsin/ml. Apart from the peptide hydrazide itself, there was no other peptide-related material visible on electrophoresis. By increasing the trypsin concentration to 5.4 mg/ml it was possible to complete the synthesis in 72 h. The recovery after separation on Sephadex G-25 was 65%. The peptide hydrazide, after separation and purification, had $m = -0.61$ at pH 6.5 [predicted value by the method of Offord (1966) is -0.61]. The amino acid composition was Asp_{4.1}Val_{1.0}Lys_{0.8}, all other amino acids less than 0.1 residue.

Electrophoretic examination indicated that the peptide hydrazide and its substituted derivatives were quantitatively reconverted into the free peptide on incubation of 20 nmol samples with 5 μ g of

trypsin in 7 μ l of 20 mM- NH_4HCO_3 /50 mM- CaCl_2 , pH 8.0, for 18 h at 37°C.

The mobilities of the substituted peptides and the known insensitivity of side-chain carboxy substituents to the action of trypsin give a strong indication that only the $\alpha\text{-CO}_2\text{H}$ group of the peptide was substituted, and that no attack took place on the four side-chain carboxy groups.

Conversion into hexapeptide Boc-hydrazide

By using the solvent system described in the Experimental section, 20% conversion into hexapeptide Boc-hydrazide was observed after a 1 week's incubation with 5.2 mg of trypsin/ml. This was increased to 70% by using 15.6 mg of trypsin/ml. The hexapeptide Boc-hydrazide, after separation by Sephadex G-25 gel filtration, had $m = -0.55$ (predicted value -0.56). It was further characterized by converting it into the corresponding unprotected hydrazide with anhydrous trifluoroacetic acid. (About 50 nmol of hexapeptide Boc-hydrazide were treated with 70 μ l of anhydrous trifluoroacetic acid for 1 h at 0°C. The trifluoroacetic acid was removed by using a water-pump vacuum apparatus). The material treated with trifluoroacetic acid had $m = -0.60$ (expected value -0.61) and, unlike the material before treatment, gave a positive result with the hydrazide stain of Barton *et al.* (1952).

Conversion into hexapeptide phenylhydrazide

By using the conditions described in the Experimental section the conversion into hexapeptide phenylhydrazide was essentially complete after 72 h incubation with 5.2 mg of trypsin/ml. The phenylhydrazide had $m = -0.55$ after separation by Sephadex G-25 gel filtration (predicted value -0.57). Electrophoretic analysis indicated that it was completely converted back into the free peptide when 20 nmol of peptide phenylhydrazide was dissolved in 20 μ l of $\text{K}_3\text{Fe}(\text{CN})_6$ (1%, w/v, in water) and the mixture left at 20°C for 30 min.

Trypsin-catalysed hydrazinolysis of performic-oxidized lysozyme

By using the conditions described in the Experimental section, hydrazinolysis was found to be overwhelmingly favoured over hydrolysis and was essentially complete after 72 h with 1.72 mg of trypsin/ml.

Conversion of a lysozyme tryptic digest into the corresponding hydrazides

Synthesis of the lysozyme tryptic peptide hydrazides was essentially complete when the same conditions as for the undigested protein were used.

The hydrazides were quantitatively converted back into the free peptides by trypsin when the same conditions as for the hexapeptide hydrazides were used, except that CaCl_2 was omitted and the reaction time was 25 min.

Trypsin-catalysed hydrazinolysis of trypsinogen

This was less successful. The hexapeptide hydrazide was visible on electrophoresis of the digest, but only in amounts approx. 10% of the expected yield. No free hexapeptide was visible, but there were a number of other ninhydrin-positive products which gave a positive reaction to the hydrazide stain of Barton *et al.* (1952).

Use of other enzymes

Hydrazinolysis of performic acid-oxidized lysozyme was attempted with the enzymes chymotrypsin, elastase and subtilisin with the conditions described above, omitting the CaCl_2 and using a final enzyme concentration of 1.72 mg/ml. In each case a series of ninhydrin-positive, hydrazide-positive spots were observed. The intensity of ninhydrin staining was essentially the same as that observed on examination of the corresponding digest carried out in the absence of hydrazine acetate (0.5%, w/v, NH_4HCO_3 , pH 8.0) at an enzyme/substrate ratio of 1:100 (w/w). The difference in the pattern of spots in the two digests was consistent with there having been complete conversion into peptide hydrazides in the former.

Other co-solvents

When glycerol was used as the co-solvent (final concn. 52%, v/v), with conditions otherwise identical with those described in the Materials and methods section for the trypsin-catalysed hydrazinolysis of lysozyme, digestion of protein was essentially complete, but hydrazinolysis was less favoured compared with hydrolysis. The ratio of peptide to peptide hydrazide was no better than 2:1. With dimethylformamide (final concn. 52%, v/v) no products of either hydrolysis or hydrazinolysis were detected.

Discussion

The successful conversion of the activation hexapeptide of bovine cationic trypsinogen is a severe test of the reaction, since this peptide has four side-chain carboxy groups as well as the one at the C-terminus. In addition, the production of a range of its hydrazide derivatives should give the opportunity to examine the usefulness of the above type of reaction as a first step in the synthesis of inhibitors specific to any given proteinase; hydrazo and analogous functions are in principle capable of specific conversion into more reactive groups such as are found in proteinase inhibitors. A peptide that had its $\alpha\text{-CO}_2\text{H}$ group specifically transformed in this way would of course be expected to be a particularly apt inhibitor for the proteinase that gave rise to it.

In general, we conclude, as did Yagisawa (1981),

that trypsin catalysis is a convenient and reliable method for the formation of both substituted and unsubstituted hydrazide derivatives of peptides. We find such catalysis to be effective for peptides of natural origin and that, in certain cases, the hydrazinolysis can be carried out simultaneously with the digestion of the parent protein. In addition, other serine proteinases appear to be effective catalysts of such reactions.

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