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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USED TO MONITOR ENZYMATIC CLEAVAGE OF PYRROLIDONE CARBOXYLIC ACID FROM REGULATORY PEPTIDES

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SUMMARY

Sequence determination of peptides blocked by amino-terminal pyrrolidone carboxylic acid (PCA) has in the past been hampered by the lack of a reliable and efficient method for removing this residue. We report here a rapid, efficient enzymatic method for removal of PCA. Reversed-phase high-performance liquid chromatography is used to monitor the reaction and to separate unblocked peptide from the reaction product. The method allows direct sequence analysis of newly purified PCA blocked peptides, eliminating the need for complicated enzymatic digestions and chromatography to determine the amino-terminal residue. Only a few micrograms of peptide are required instead of the several milligrams needed in the past.

INTRODUCTION

Many important naturally occurring mammalian peptides such as gastrins, neurotensin and releasing factors, as well as amphibian peptides such as caerulein, bombesin, ranatensin and litorin, contain an amino-terminal pyrrolidone carboxylic acid (PCA) residue. These residues are formed when amino-terminal glutamyl or glutaminyl residues cyclise with their α-amino groups spontaneously, or in enzymatically catalysed reactions. This leaves the α-amino groups unavailable for reaction with phenyl isothiocyanate so that peptides with an amino-terminal PCA residue cannot be sequenced by the Edmán procedure. The literature on structures of PCA-blocked peptides illustrates the difficulties that are encountered if this group cannot be easily removed. Thus the sequence of a neuropeptide found in hydra and in the brain of man, rat, cow and pig was deduced only because the PCA residue opened up during the last steps of purification in a portion of the peptide[1]. The sequence of caerulein was deduced when partial acid hydrolysis produced a peptide which contained two residues of glutamic acid after complete hydrolysis, and which was shown to co-

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migrate with PCA-Gln in several electrophoretic systems[2]. The sequence analysis of the heptadecapeptide gastrin raised similar problems and the structure was deduced from data after papain and carboxypeptidase digestions, and amino acid analysis of the remaining tripeptide PCA-Gly-Pro[3].

Removal of amino-terminal blocking PCA residues would have rendered all of these peptides available for sequence determination through Edman procedures. Chemical and enzymatic methods have been developed for the removal of this residue[4–6], but incomplete reaction or cleavage of peptide bonds at other than the amino-terminus have complicated the reactions. This report describes an enzymatic method which monitors the extent of PCA removal, separates unblocked peptide from the reaction products and is suitable for unblocking a few nanomoles of peptide.

EXPERIMENTAL

Equipment

High-performance liquid chromatography (HPLC). An Altex Scientific (Berkeley, CA, U.S.A.) Model 312MP liquid chromatograph equipped with a 210 sample injector and an Altex-Hitachi 155-40 variable-wavelength detector was employed. All separations were performed on a reversed-phase C_{18} column (Waters Bondapak, 10 μm, 3.9 × 300 mm).

Amino acid analysis. A Beckman 118 CL amino-acid analyser was used; the ninhydrin-derivatised amino acids were detected at 570 and 440 nm, and the peaks integrated in a Beckman 126 data system for quantitation.

Incubation conditions

Reactions were incubated at a constant temperature of 40°C using a solid heating block (Lab Line Inc.).

Reagents and chemicals

PCA peptidase from *Bacillus amyloliquefaciens* was a generous gift from Professor D. Tsuru; enzyme from calf liver was purchased from Boehringer (Mannheim, G.F.R.). Natural human G17 sulphated and synthetic human G34 unsulphated were gifts from Professor R. A. Gregory and Dr. H. J. Tracy. Synthetic bombesin 14 was obtained from Farmitalia (Milan, Italy) and from Peninsula (San Carlos, CA, U.S.A.). HPLC-grade water and acetonitrile were purchased from Baker (Philipsburg, NJ, U.S.A.). Sequenal grade trifluoroacetic acid (TFA) and constant-boiling hydrochloric acid were from Pierce (Rockford, IL, U.S.A.). EDTA and ammonium bicarbonate were from Mallinckrodt (St. Louis, MO, U.S.A.) and mercaptoethanol and mercaptoacetic acid were from Sigma (St. Louis, MO, U.S.A).

Methods

Incubation conditions. All enzyme incubations were performed in 0.13 *M* ammonium bicarbonate containing EDTA (3 m*M*) and mercaptoethanol (3 m*M*). Glass tubes were used throughout and the reaction temperature was maintained at 40°C using a solid heating block.

Natural human G17. Peptide (0.25 mg) was dissolved in 0.5 ml of incubation buffer and treated with 0.002, 0.006 or 0.02 units of Tsuru enzyme or 0.002 U of

Boehringer enzyme. Aliquots of 20 μl were removed at intervals from 0 to 120 min and added to 20 μl of TFA (0.28 *M*) prior to chromatography.

Synthetic human G34. Peptide (0.15 mg) was dissolved in 0.3 ml of buffer and treated with 0.02 U of Tsuru enzyme. Samples of 10 μl were removed at intervals from 0 to 60 min and added to an equal volume of 0.28 *M* TFA.

Synthetic bombesin 14. Peptides (50 μg) from Farmitalia and from Peninsula were separately dissolved in 0.3 ml of incubation buffer and treated with 0.02 U of Tsuru or Boehringer enzyme. Samples of 10 μl were removed at intervals from 0 to 180 min and neutralised as above with TFA.

Chromatography. Samples of TFA-treated reaction mixtures were chromatographed isocratically on a reversed-phase C_{18} Bondapak 10 μm column equilibrated with TFA(0.1 %)-acetonitrile. Injection volumes were 10–40 μl. Buffer systems employed were: for HG17, TFA–acetonitrile (65:35); for HG34, TFA–acetonitrile (68:32); and for B14, TFA–acetonitrile (74:26). The degree of PCA removal was calculated by integrating the areas under the peaks of absorbance at 206 or 280 nm.

Amino acid analysis. Pools of HPLC-eluted peptide corresponding to peaks of absorbance were lyophilized in a hydrolysis tube, 1 ml of 6 *M* hydrochloric acid containing 0.02 % mercaptoacetic acid was added, and the tube was sealed under vacuum. The tube was heated at 110°C for 20 h, opened, the hydrochloric acid was removed by lyophilization and the contents were dissolved in Beckman buffer for amino-acid analysis. The sample was applied to a Beckman 118 CL amino acid analyser and the amino acids were eluted according to the method of Spackman[7].

RESULTS AND DISCUSSION

After reversed-phase HPLC, unreacted human G17 was resolved as a single major absorbance peak at 280 nm with a retention time of 6.0 min. As the reaction progressed, there appeared a less well retained peak (retention time 4.0 min), together with a proportionate decrease in the size of the initial HG17 peak (Fig. 1). Amino acid analysis of the initial 6.0-min peak confirmed that it had the amino acid composition of HG17, whilst the earlier post-reaction peak differed only in that it contained one less glutamyl residue (Table I). The reaction was dependent on both time and enzyme concentration; both enzyme preparations had similar kinetics with the Boehringer preparation having somewhat higher activity per unit enzyme (Fig. 2). At the highest enzyme concentration used with G17 (0.04 U/ml) approximately 100 % removal of PCA was achieved in 30 min. If the reaction temperature was maintained at 18°C rather than the routine 40°C then there was virtually no conversion, even after 24 h.

Rapid removal of PCA from synthetic human G34 was also achieved such that, at an enzyme concentration of 0.06 U/ml, 100 % PCA removal took only 10 min (Fig. 3). However, the reaction with synthetic bombesins was much slower, and after incubation for 3 h with enzyme at 0.06 U/ml only 50 % conversion was realised (Fig. 3). Using a series of synthetic dipeptides, Doolittle[4] showed that the rate of cleavage depends upon the residue adjacent to the PCA, and we have confirmed that this relationship also holds for naturally occurring peptides. Thus bombesin (PCA-Gln) was cleaved much more slowly than human G17 (PCA-Gly) or human G34 (PCA-Leu). Other factors such as teriary structure may also be important in determining the rate of PCA cleavage. Both HG17 and HG34 and the Farmitalia B14 chromato-

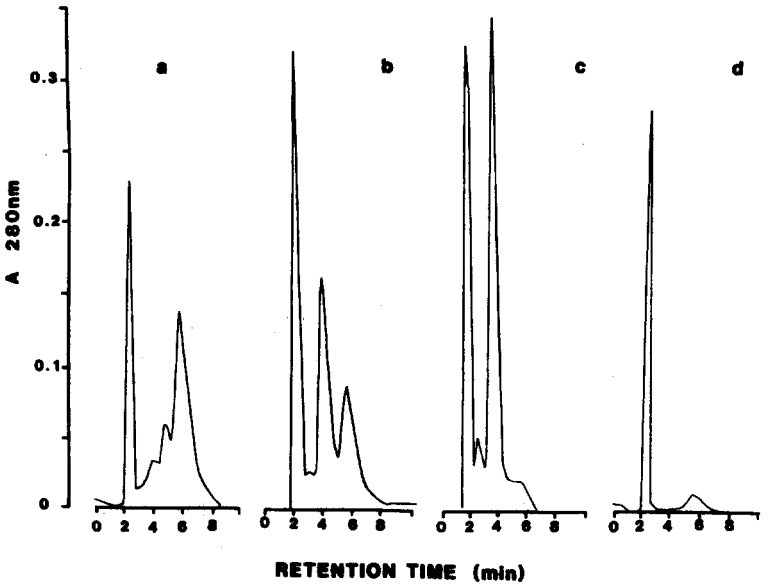

Fig. 1. HPLC elution profiles of natural human G17 before and after digestion with PCA peptidase from *Bacillus amyloliquefaciens* (a) before reaction, (b) after 5 min reaction with enzyme, (c) after 20 min reaction with enzyme, (d) blank run containing enzyme but no peptide. See methods for chromatographic details.

TABLE I

AMINO ACID ANALYSIS OF HUMAN G17 BEFORE AND AFTER DIGESTION WITH PCA PEPTIDASE

Figures in parentheses indicate integral values assigned.

Amino acid	*Pre-digest*	*Post-digest*
Asp	1.0(1)	1.0(1)
Thr	0(−)	0(−)
Ser	0(−)	0(−)
Glu	5.8(6)	5.1(5)
Pro	0.9(1)	0.9(1)
Gly	1.6(2)	1.8(2)
Ala	1.0(1)	1.0(1)
Val	0(−)	0(−)
Met	1.0(1)	1.0(1)
Ile	0(−)	0(−)
Leu	0.9(1)	0.9(1)
Tyr	0.9(1)	0.9(1)
Phe	0.9(1)	0.9(1)
His	0(−)	0.1(−)
Lys	0.2(−)	0.3(−)
Arg	0(−)	0(−)
Trp*	1.3(2)	1.3(2)

* Tryptophan is incompletely recovered under the acid hydrolysis conditions employed here.

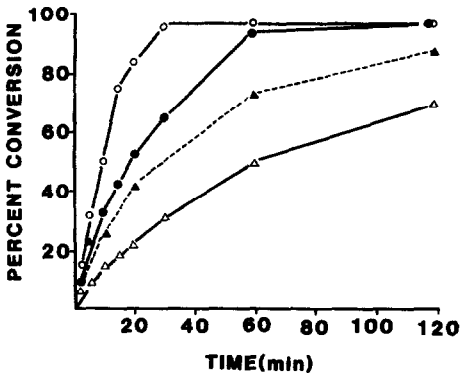

Fig. 2. Rate of conversion of natural human G17 into 2-17 hG17, by incubation with PCA peptidases: ○, Tsuru enzyme (0.04 U/ml); ●, Tsuru enzyme (0.013 U/ml); △, Tsuru enzyme (0.004 U/ml); ▲, Boehringer enzyme (0.004 U/ml).

graphed initially as a single peak after reversed-phase HPLC and, after PCA peptidase treatment, gave rise to one further, less well retained, peak (Fig. 1). However, unreacted Peninsula B14 initially chromatographed as a double peak of absorbance at 206 nm and, after PCA peptidase treatment, gave rise to two further peaks, each with a reduced retention time relative to the first peaks. If the Peninsula B14 was incubated for 120 min in reaction buffer (containing 3 m*M* mercaptoethanol), then only a single peak was evident after reversed-phase HPLC of the unreacted peptide, which had the same retention time as the Farmitalia B14. This finding was unexpected and suggested to us that a proportion of the Peninsula B14 may have contained partially oxidized methionine residues (methionine sulphoxide) which were reduced in the presence of mercaptoethanol. Similar reduction of methionine sulphoxide to methionine has been described using the reducing agent N-methyl mercaptoacetamide[8], and this has been applied to the reduction of partially oxidized vasoactive intestinal peptide (VIP) and cholecystokinin (CCK) such that full biological activity was restored to the latter peptide[9].

The PCA removals described above were performed on 25–150 nmol of pep-

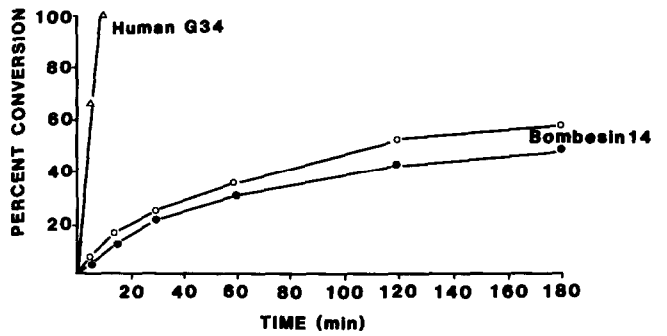

Fig. 3. Rate of removal of PCA from synthetic human G34 and synthetic bombesin 14: △, human G34 (Tsuru enzyme, 0.06 U/ml); ○, Farmitalia bombesin 14 (Boehringer enzyme, 0.06 U/ml); ●, Farmitalia bombesin 14 (Tsuru enzyme, 0.06 U/ml).

tide, but we have also used this system to remove PCA from approximately 4 nmol of newly isolated rat heptadecapeptide gastrin[10]. As with the human G17, the PCA-peptidase-reacted rat gastrin was separated from the unreacted peptide, and the 2-17 rat gastrin proved suitable for microsequence analysis by the Edman procedure.

In summary, we have described a method that monitors the extent of enzymatically induced PCA removal, separates the unblocked peptide from the reaction products and is capable of unblocking just a few micrograms of peptide. The unblocked product is suitable for Edman degredation procedures used in sequence analysis. This method should allow direct sequence analysis of newly purified PCA blocked peptides and should eliminate the necessity for complicated enzymatic digestions and chromatography to determine the amino-terminal residue. This will help the performance of sequence analysis on a few micrograms of purified peptide instead of the several milligrams that have been required in the past.

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