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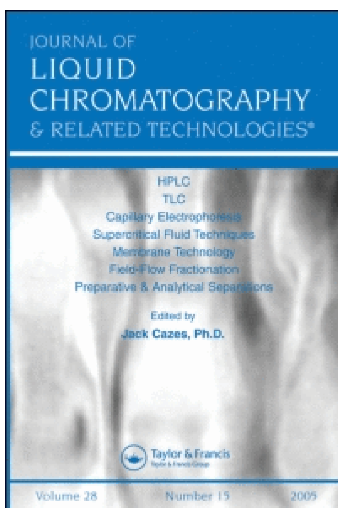

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Monitoring of Protease-Catalyzed Peptide Synthesis by High Performance Liquid Chromatography

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(7), 1121-1134 (1981)

MONITORING OF PROTEASE-CATALYZED PEPTIDE SYNTHESIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography (HPLC) on prepacked silica gel columns has been applied to the separation of closely related protected peptides and amino acids. In the course of a protease-catalyzed synthesis of Leu-enkephalin this chromatographic technique was found to be a valuable tool to rapidly and reliably characterize the outcome of enzymatic reactions, the nature of which was often difficult to be predicted. Stepwise gradient elution was employed to enable fractionation of mixture components, which covered only a short polarity range. The solvent systems composed of dichloromethane, anhydrous ethanol and acetic acid were mixed in such ratios so as to provide completely resolved peaks for the sample problems studied so far. Enzymatically prepared compounds and their chemically synthesized, authentic analogues were cochromatographed to enable the assignment of the eluted peaks. The provisional identification thus obtained could finally be established for all compounds under study by standard methods for chemical analysis.

INTRODUCTION

During the last years peptide bond formation by reversal of enzymic proteolysis has become a promising approach to preparative-scale peptide synthesis. Due to the specificity of the proteases serving as catalysts, undesired side-reactions often found

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during conventional chemical syntheses are eliminated. In spite of these advantageous features, the enzymic procedure still lacks the general applicability, because it is often difficult to foresee whether and to what extent the required peptides are going to be formed. Furthermore, a priori proteolytic cleavage of the preexisting peptide bonds may give rise to undesired, and often truncated peptides. Therefore, one cannot be sure, that the amino acid composition of the products is identical to that of the target peptide. As a consequence, these ambiguities make a methodical design of synthesis more difficult or even render it a mere trial and error procedure. Thus a rapid but yet critical evaluation of the nature of the products resulting from enzymatic reactions becomes highly desirable.

The application of high performance liquid chromatography (HPLC) to the separation of both underivatized and protected peptides either in reversed or in normal phase modes has gained wide acceptance during the past few years (1-7).

While studying the capability of individual proteases to serve as catalysts during the synthesis of Leu-enkephalin, the speed and high resolving power of HPLC on prepacked silica gel columns provided for a rapid and reliable characterization of the outcome of enzymatic reactions. The results of chromatographic analyses thus enabled us to judge readily and conclusively on the prospects of a given synthetic pathway, in particular, to decide whether or not it should be continued.

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MATERIALS AND METHODS

HPLC was carried out on Li Chroprep Si 60 columns (24 cm x 1 cm I.D., 40 - 63 μm particle size) (Merck, Darmstadt, F.R.G.) using a Constametric II G delivery system (Milton Roy, Riviera Beach, Fl.). Peptide or amino acid samples were dissolved in the starting eluant, filtered, and loaded via a teflon 4-way rotary valve, fitted with a 0.5-ml injection loop (Latek, Heidelberg, F.R.G.). The columns were eluted in a stepwise mode by solvent systems of different polarity at ambient temperature in upward flow operation. A constant flow-rate of 2 ml/min was maintained. Elutions were followed continuously by monitoring UV absorbance at 254 nm using a Uvicord II photometer (LKB, Bromma, Sweden) which was connected to an LKB Biocal recorder. The eluate was collected at 2-min intervals using an LKB Ultrorac fraction collector.

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All solvents used were analytical-grade. Dichloromethane and acetic acid were purchased from Baker (Groß-Gerau, F.R.G.), and anhydrous ethanol was obtained from Merck. Prior to use the solvents were degassed, mixed on the required volume-to-volume basis, filtered and degassed again. All columns were equilibrated with the starting eluant for at least one hour. The solvent systems were stirred during equilibration and elution.

The preparation of the amino acid and peptide derivatives dealt with in this report has been described elsewhere (8, 9). The enzymatically prepared compounds to be analyzed were chromatographed in

the presence of chemically prepared references of known composition for purposes of qualitative comparison. The peaks in HPLC effluents were further characterized by elemental analysis (Fa. Beller, Göttingen, F.R.G.), and by amino acid analysis of acidic hydrolysates performed on a Biotronik model LC 6000 E analyzer.

RESULTS AND DISCUSSION

The protected peptides and amino acids described below were obtained in the course of a protease-catalyzed synthesis of Leu-enkephalin (8, 9). Fractionation via HPLC enabled a rapid judgement on the identity or non-identity of the enzymatically derived products to the target compounds, authentic analogues of which were used as internal standards. The tentative positive or negative identification of each chromatographic peak thus obtained was finally confirmed by amino acid and/or elemental analyses.

Under the conditions prevailing during the chromatographic processes those compounds carrying free amino- or carboxyl-groups were retained on the columns.

According to the initial design of synthesis, the papain-mediated coupling of Boc-Tyr(Bzl)-Gly-Gly-OH (10) and H-Phe-Leu-N_2H_2Ph was supposed to be the last step leading to a protected Leu-enkephalin. Thin-layer chromatographic data suggested an unexpected outcome of the enzymatic reaction, but did not permit any definite conclusion. Therefore, the product of unknown composition was submitted to HPLC on silica gel 60 columns both in the presence and

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in the absence of a reference pentapeptide. The resulting chromatographic data convincingly demonstrated that the compound under study did not correspond to the authentic pentapeptide (Fig. 1). Elemental and amino acid analysis revealed the chemical structure of the unknown compound to be Boc-Tyr(Bzl)-Gly-Phe-Leu-N_2H_2Ph. $C_{44}H_{54}N_6O_7$: Found: C, 68.07; H 6.75; N, 10.87; Calcd.: C, 67.83; H, 6.99; N, 10.79. Amino acid composition: Gly, 1.00(1); Leu, 0.97(1); Tyr, 0.91(1); Phe, 1.04(1).

As illustrated by the elution profile (Fig. 1), the pentapeptide was absorbed more strongly to the silica support than the

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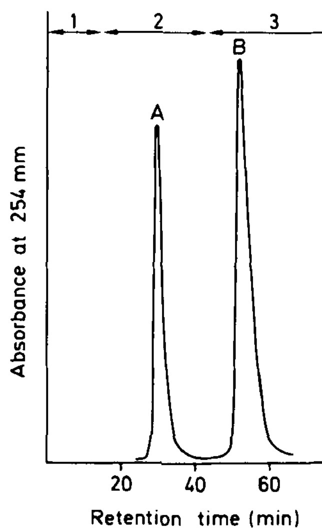
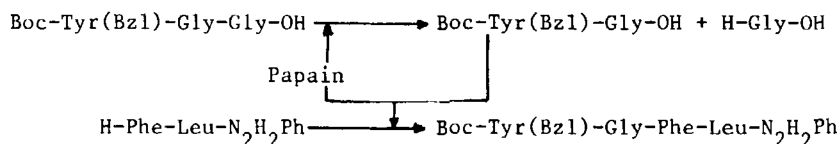


FIGURE 1. Separation of Boc-Tyr(Bzl)-Gly-Phe-Leu-N_2H_2Ph (A) and Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N_2H_2Ph (B) on a Li Chroprep Si 60 (24 x 1 cm) column. Flow-rate 2 ml/min. Mobile phase (three-step gradient): (1) dichloromethane, (2) dichloromethane - anhydrous ethanol - acetic acid (100:3.5:3), (3) dichloromethane - anhydrous ethanol - acetic acid (100:5:5).

tetrapeptide. This behavior can be explained by the presence of an additional polarizable peptide bond which renders the pentapeptide more polar with respect to the tetrapeptide.

The papain-mediated reaction presumably proceeded according to the following scheme:



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This conception was supported by the finding, that free glycine, which could be traced by thin-layer chromatography, was released during the enzymatic process. In the light of these results, the above mentioned synthetic route could be considered a failure.

The desired pentapeptide Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N_2H_2Ph was finally prepared by reacting Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt and H-Leu-N_2H_2Ph in the presence of α-chymotrypsin. The progress of this reaction could be conveniently monitored via HPLC, because the tetrapeptide donor ester and the resulting pentapeptide were selectively retained during their passage through a silica gel column (Fig. 2). The enzymatically synthesized pentapeptide occurred in the elution profile in the exact position of the chemically prepared reference pentapeptide. Elemental analysis: Found: C, 66.32; H, 6.96; N, 11.71; $C_{46}H_{57}N_7O_8$ requires: C, 66.09; H, 6.87; N, 11.73. Amino acid analysis: Gly, 2.00(2); Leu, 0.96(1); Tyr, 0.85(1); Phe, 1.02(1).

The above tetrapeptide ethylester was obtained by bromosuccinimide treatment of the tetrapeptide Boc-Tyr(Bzl)-Gly-Gly-Phe-N_2H_2Ph followed by the addition of anhydrous ethanol (11). Notwithstanding the fact, that the two tetrapeptides solely differed with respect to the carboxyl protection, supervision of this reaction was readily accomplished by HPLC. As illustrated in Figure 2, the chromatographic peaks of the respective tetrapeptides could be completely resolved. The prolonged elution time of the tetrapeptide phenylhydrazide was presumably caused by the more polar

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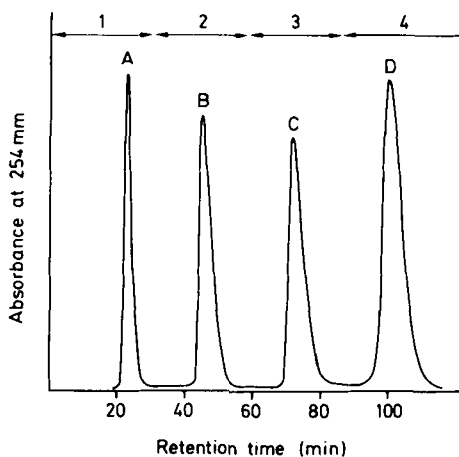
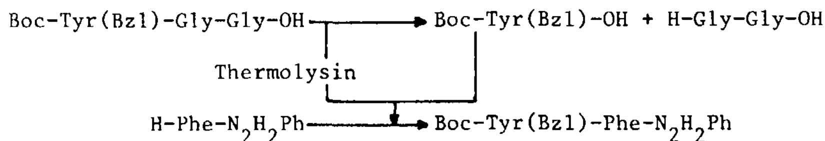


FIGURE 2. Analysis of a mixture of four derivatized peptides under the conditions of a four-step gradient. Column: Li Chroprep Si 60 (24 x 1 cm). Flow-rate 2 ml/min. Mobile phase: (1) dichloromethane - anhydrous ethanol - acetic acid (100:1:1), (2) dichloromethane - anhydrous ethanol - acetic acid (100:3:3), (3) dichloromethane - anhydrous ethanol - acetic acid (100:4:4), (4) dichloromethane - anhydrous ethanol - acetic acid (100:5:5). Components: Boc-Tyr(Bzl)-Phe-N_2H_2Ph (A), Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt (B), Boc-Tyr(Bzl)-Gly-Gly-Phe-N_2H_2Ph (C), Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N_2N_2Ph (D).

character of the phenylhydrazide protecting group relative to the ethyl ester moiety. Elemental analyses of the above peptides gave the following results: Tetrapeptide ethyl ester; $C_{36}H_{44}N_{4}O_{8}$: Found: C, 65.68; H, 7.00; N, 8.52; Calcd.: C, 65.44; H, 6.71; N, 8.48. Tetrapeptide phenylhydrazide; $C_{40}H_{46}N_{6}O_{7}$: Found: C 66.48; H, 6.39; N, 11.70; Calcd.: C, 66.47; H, 6.42; N, 11.63.

Although the tetrapeptide Boc-Tyr(Bzl)-Gly-Gly-Phe-N_2H_2Ph could be synthesized by means of papain-catalyzed coupling of Boc-Tyr(Bzl)-Gly-OH and H-Gly-Phe-N_2H_2Ph, we tried, however, to prepare it from Boc-Tyr(Bzl)-Gly-Gly-OH and H-Phe-N_2H_2Ph in the presence of thermolysin. The resulting product was chromatographed on a silica gel column. Its non-identity to the authentic tetrapeptide, which had served as an internal standard, could be decisively demonstrated (Fig. 2). Elemental and amino acid analyses proved the product of interest to be Boc-Tyr(Bzl)-Phe-N_2H_2Ph. $C_{36}H_{40}N_{4}O_{5}$: Found: C, 71.06; H, 6.37; N, 9.43; Calcd.: C, 71.03; H, 6.62; N, 9.20. Amino acid composition: Gly, negative; Tyr, 0.90(1); Phe, 1.00(1).

In addition to the dipeptide Boc-Tyr(Bzl)-Phe-N_2H_2Ph, the underivatized dipeptide H-Gly-Gly-OH was formed during the enzymatic reaction. The thermolysin-controlled reaction presumably proceeded according to the following scheme:



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To exemplify the resolving power of the HPLC technique four out of five protected peptides described above have been cochromatographed (Fig. 2). The significant contribution of peptide bonds to the overall adsorption affinity of a given molecule to the polar adsorbant was well demonstrated by the elution behavior of the respective sample components. With regard to the polarizable groups, which almost entirely determine the binding strength of a solute to a polar support, the fractionated phenylhydrazide peptides differed from each other only in the number of their peptide bonds. As illustrated in Figure 2, the elution order of the phenylhydrazide compounds was indeed directly related to the number of their peptide bonds.

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The tripeptide Boc-Gly-Phe-Leu-N_2H_2Ph, the deacylated form of which served as amino component during a papain-catalyzed synthesis of a derivatized Leu-enkephalin (8), was obtained by α-chymotrypsin-mediated coupling of Boc-Gly-Phe-OEt and H-Leu-N_2H_2Ph. This reaction could be easily monitored by HPLC because the dipeptide ester and the tripeptide phenylhydrazide were easily separated on silica gel columns (Fig. 3). Peak identities were confirmed as follows: Boc-Gly-Phe-OEt: Found: C, 61.86; H, 7.18; N, 7.80; $C_{18}H_{26}N_2O_5$ requires: C, 61.71; H, 7.48; N, 8.00. Boc-Gly-Phe-Leu-N_2H_2Ph: Found: C, 64.40; H, 6.91; N, 13.42; $C_{28}H_{39}N_5O_5$ requires: C, 63.98; H, 7.48; N, 13.32.

The attempt to synthesize the above tripeptide via peptide bond formation between Boc-Gly-Phe-OH and H-Leu-N_2H_2Ph in the presence of papain resulted in a product, the non-identity of which

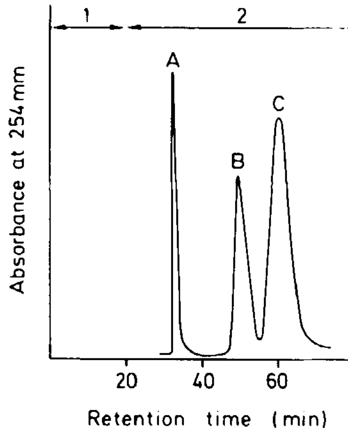

FIGURE 3. Elution profile of a mixture of protected peptides obtained from a Li Chroprep Si 60 (24 x 1 cm) column with a two-step gradient. Mobile phase: (1) dichloromethane, (2) dichloromethane - anhydrous ethanol - acetic acid (100:2:2). Flow-rate 2 ml/min. Peak A corresponds to Boc-Gly-Phe-OEt, peak B corresponds to Boc-Gly-Leu-N_2H_2Ph, peak C corresponds to Boc-Gly-Phe-Leu-N_2H_2Ph.

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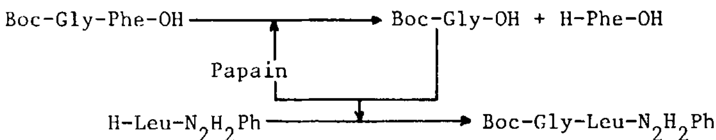
to the desired peptide was readily proved by HPLC. As illustrated by the elution diagram (Fig. 3) the retention time of the product under study - its chemical structure was determined to be Boc-Gly-Leu-N_2H_2Ph - did not correspond to that of the authentic tripeptide. Elemental analysis (Boc-Gly-Leu-N_2H_2Ph; $C_{19}H_{30}N_4O_4$): Found: C, 60.23; H, 7.96; N, 14.78; Calcd.: C, 60.30; H, 7.99; N, 14.80. Amino acid analysis: Gly, 1.00(1); Leu, 0.96(1); Phe, negative.

The enzymatic reaction probably proceeded according to the following scheme:



The dipeptide Boc-Tyr-Gly-N_2H_2Ph, a precursor of Boc-Tyr(Bzl)-Gly-OH (vide supra), was obtained from Boc-Tyr-OEt and H-Gly-N_2H_2Ph by α-chymotrypsin catalysis. The difference in chromatographic mobility on silica gel columns of the donor ester and of the resulting dipeptide (Fig. 4) enabled a rapid control of the progress of this reaction by means of HPLC. The chromatographic data were confirmed by elemental analyses: (Boc-Tyr-OEt; $C_{16}H_{23}NO_5$): Found: C, 61.75; H, 7.39; N, 4.49; Calcd.: C, 62.13; H, 7.49; N, 4.53. (Boc-Tyr-Gly-N_2H_2Ph; $C_{22}H_{28}N_4O_5$): Found: C, 61.43; H, 6.49; N, 13.16; Calcd.: C, 61.68; H, 6.59; N, 13.08.

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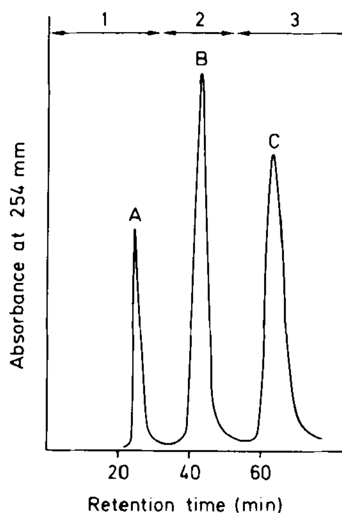
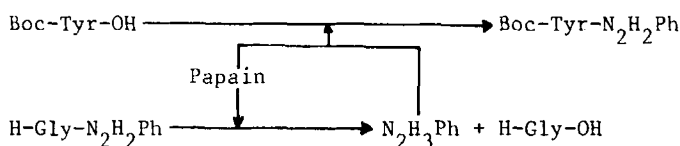


FIGURE 4. Chromatogram obtained during the fractionation of Boc-Tyr-OEt (A), Boc-Tyr-N_2H_2Ph (B) and Boc-Tyr-Gly-N_2H_2Ph (C) on a Li Chroprep Si 60 (24 x 1 cm) column. Flow-rate 2 ml/min. Mobile phase (three-step gradient): (1) dichloromethane - anhydrous ethanol - acetic acid (100:1:1), (2) dichloromethane - anhydrous ethanol - acetic acid (100:2:2), (3) dichloromethane - anhydrous ethanol - acetic acid (100:5:5).

An attempt to prepare the dipeptide Boc-Tyr-Gly-N_2H_2Ph by papain-catalyzed peptide bond formation between Boc-Tyr-OH and H-Gly-N_2H_2Ph furnished a product, which did not match the retention characteristics on silica supports of the target peptide (Fig. 4). According to elemental and amino acid analyses the compound of unknown composition was shown to be Boc-Tyr-N_2H_2Ph. $C_{20}H_{25}N_3O_4$: Found: C, 64.32; H, 6.84; N, 11.52; Calcd.: C, 64.64; H, 6.78; N, 11.36. Amino acid analysis: Gly, negative; Tyr, positive.

The papain-mediated reaction presumably proceeded via transamidation as outlined in the following scheme:



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The elution profiles of Boc-Tyr-OEt, Boc-Tyr-N_2H_2Ph and Boc-Tyr-Gly-N_2H_2Ph (Fig. 4) exemplify the retention behavior on a hydrophilic support of a given compound with respect to the type and the number of its polarizable groups. The adsorption affinity of the acylated tyrosine phenylhydrazide, which already exceeded that of the corresponding ethyl ester derivative, was further strengthened by an additional peptide bond contributed by the insertion of a glycine residue.

The examples reported above are to demonstrate the usefulness of HPLC on silica gel columns for the rapid and reliable characterization of closely related protected peptides and amino

acids. The high resolving power and the ease of operation made this technique ideally suited for routine analyses of enzymatically prepared compounds of rather lipophilic nature. As the affinity of the adsorptives for hydrophilic adsorbents is almost entirely determined by polar interactions, the individual components of a sample were eluted at different rates depending on their degree of polarity. Consequently, the protected peptides and amino acids dealt with in this paper could be separated according to the differences in number and diversity of their polarizable groups. The eluent systems, covering a rather narrow polarity range, were composed of dichloromethane, anhydrous ethanol and acetic acid in different ratios. They sufficiently provided both elutive power and selectivity so as to enable complete resolution of amino acids and peptides varying by as little as a single protecting group or by only one amino acid residue in chain length.

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