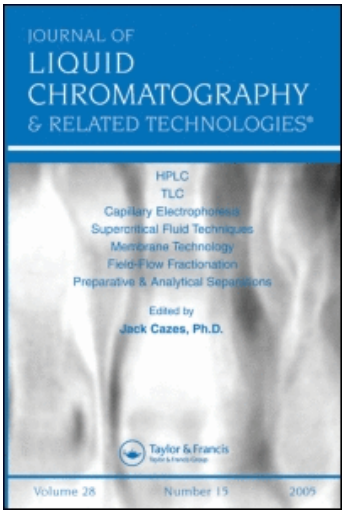

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Rapid Characterization by Thin-Layer Chromatography of Amino Acid and Peptide Derivatives Enzymically Prepared During Protease-Mediated Peptide Synthesis

Willi Kullmann[a]
[a] Max-Planck-Institut für Biophysikalische Chemie Am Faßberg, Göttingen-Nikolausberg, Germany

To cite this Article Kullmann, Willi(1981) 'Rapid Characterization by Thin-Layer Chromatography of Amino Acid and Peptide Derivatives Enzymically Prepared During Protease-Mediated Peptide Synthesis', Journal of Liquid Chromatography & Related Technologies, 4: 11, 1947 — 1959
To link to this Article: DOI: 10.1080/01483918108067554
URL: http://dx.doi.org/10.1080/01483918108067554

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(11), 1947-1959 (1981)

RAPID CHARACTERIZATION BY THIN-LAYER CHROMATOGRAPHY OF AMINO ACID AND PEPTIDE DERIVATIVES ENZYMICALLY PREPARED DURING PROTEASE-MEDIATED PEPTIDE SYNTHESIS.

Willi Kullmann
Max-Planck-Institut für Biophysikalische Chemie
Am Faßberg
3400 Göttingen-Nikolausberg
Germany

ABSTRACT

Thin-layer chromatographic position matching combined with color matching appeared to be a convenient method for characterization of the products of individual synthetic reactions during the protease-catalyzed synthesis of Leu- and Met-enkephalin.

INTRODUCTION

Recently, we have reported two alternative pathways to the protease-catalyzed synthesis of Leu- and Met-enkephalin (1). In the course of this study, several promising pathways had to be discarded or modified. The specificity of the proteases used as catalysts was often not sharp enough to permit predictions whether and to what extent the desired compounds were going to be formed (2). These imponderables required permanent supervision of the progress of enzymic reactions and a criti-

cal evaluation of the nature of the resulting products.

Therefore, the outcome of each enzymic reaction was routinely monitored by thin-layer chromatography. The chromatograms were developed in two solvent systems, which usually provided for sufficient resolving power to ensure rapid discrimination of the resulting products. In several cases, however, products derived from enzymic reactions moved to positions on the chromatoplates difficult to be distinguished from those of the reactants. The overlapping of the spots thus formed prevented an unambiguous assignment by position matching of the enzymically prepared compounds to their chemically synthetized analogs, which served as reference substances.

In this work a method has been developed, which allows a valid assignment of the products in question by a combination of position and color matching. The procedure is based on groupspecific chromogenic reagents and on the capacity of ninhydrin to develop distinct transient colors when reacted with various amino acid and peptide derivatives.

MATERIALS AND METHODS

All solvents used in this work were reagent grade. Ninhydrin and sodium nitroprusside were purchased from

Merck, Darmstadt, F.R.G., 1-naphthol and bromine were obtained from Fluka, Switzerland. Amino acid analyses of acidic hydrolysates were performed on a Biotronik analyzer LC 6000 E. Elemental analyses were carried out by Fa. Beller, Göttingen, F.R.G.

Ninhydrin spray was prepared by dissolving 1.0 g of ninhydrin in 25 ml of acetone-pyridine-acetic acid (485:15:10)(v/v/v).

1-naphthol - hypobromite (Sakaguchi-reagent)(3):

Solution 1: 50 ml of a 16% urea solution were combined with 10 ml of a 0.2% ethanolic 1-naphthol solution.

Solution 2: Bromine (0.5 ml) was added to 50 ml of a 5% sodium hydroxide solution.

To visualize the spots the plates were first sprayed with solution 1, then dried for 1 h at 40°C and subsequently sprayed with solution 2. Sakaguchi-positive substances gave pink-colored spots.

Sodium nitroprusside-reaction (3):

The plates were sprayed with a 2% aqueous sodium nitroprusside solution. Bluish-grey spots were formed in the presence of reactive compounds on exposure to ammonia vapor. Dilution series of chemically prepared amino acid and peptide derivatives ranging from 1.0 to 10.0 µg were applied in 1.5 µl quantities of methanolic solution to precoated Silia Gel 60 plates (layer

thickness 0.25 mm)(Merck) and were run alongside the samples to be analyzed for purposes of quantitative and qualitative comparison. The plates were developed both in the solvent system chloroform-methanol (3:1)(v/v)(A) and in the system chloroform-methanol-acetic acid (45:4:1)(v/v/v)(B). The chromatograms were allowed to run in a saturated tank, the walls of which were covered with filter paper, until the solvent front had advanced a sufficient distance (18 cm), and were then air-dried to remove the solvents. In the case of ninhydrin treatment, the plates were heated for 20 min at 110°C to deacylate the α-amino group of the protected amino acids or peptides and immediately sprayed while still hot. An atomizer connected to a container filled with propellant gas (Pierce Chem.Co., Rockfort, Ill., U.S.A.) at a distant of 30 cm from the plate was used to achieve even dispersion of the freshly prepared spray.

RESULTS AND DISCUSSION

As a consequence of the abovementioned uncertainties in the outcome of protease-catalyzed peptide synthesis it is rather difficult to design a synthetic pathway. One is therefore often confined to a mere trial and error procedure. As a matter of course, it

is favorable in this situation to quickly establish whether or not a desired compound is formed under the prevailing conditions. Thin-layer chromatography was found to be a valuable tool to rapidly facilitate a provisional but nevertheless valid estimation of the progress and the results of the individual enzymic reaction. The solvent system chloroform-methanol (3:1) enabled a rapid and distinct separation of fully protected products from partially deprotected reactants. Due to their low adsorptive affinity to the polar adsorbent (silica gel) and their considerable solubility in the eluotropic solvent, the hydrophobic products displayed high R_F-values whereas the educts, the free α-carboxyl or α-amino groups of which contributed to their more hydrophilic character, exhibited medium R_F-values.

This was not the case when N^{α}-acylated amino acid or peptide ethyl esters served as carboxyl components in enzymic reactions. The ethyl ester derivatives were used, because they were shown to be excellent donor-substrates for α-chymotrypsin-mediated peptide bond formation (4). But they moved to positions on the chromatograms so close to those occupied by the resultant products that there were serious possibilities of confusion. The solvent system chloroform-methanol-acetic acid (45:4:1) indeed enabled satisfactory resolution

with regard to products and esterified reactants, but it did not always separate the products from the degradation products resulting from enzymic ester hydrolysis of the donor-substrates. Therefore this solvent system was also of only limited use for monitoring α-chymotrypsin-catalyzed reactions. However, it was useful to control the homogeneity of purified compounds.

The phenylhydrazide protection of the amino components was originally chosen, because it promotes peptide bond formation by strengthening the nucleophilicity of the α-amino group and by decreasing the solubility of the resulting products, which were to be precipitated (1,2). In addition, this protecting group now opened up a suitable means to distinguish between the newly formed N^{α}-acylated peptide phenylhydrazides and the esterified reactants. The finding, that phenylhydrazide derivatives gave color reactions both with hypobromite - 1-naphthol (Sakaguchi-reagent) and with sodium nitroprusside whereas ethyl ester derivatives remained unaffected, enabled a rapid, convenient supervision of the following α-chymotrypsin-catalyzed reactions (details are given in Table 1):

Boc-Phe-OEt (5) + H-Leu-N_2H_2Ph

⟶ Boc-Phe-Leu-N_2H_2Ph

Boc-Gly-Phe-OEt + H-Leu-N_2H_2Ph

⟶ Boc-Gly-Phe-Leu-N_2H_2Ph

Boc-Gly-Phe-OEt + H-Met-N_2H_2Ph

⟶ Boc-Gly-Phe-Met-N_2H_2Ph

Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt + H-Leu-N_2H_2Ph

⟶ Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N_2H_2Ph

The partially deprotected amino components could be easily separated owing to their differing R_F-values.

Visually comparing the color development resulting from ninhydrin-treatment provided another approach to distinguish ethyl ester from phenylhydrazide derivatives. The latter initially produced faintly yellow spots which gradually darkened whereas the former compounds quickly displayed dull colors (Table 1).

The usefulness of color matching could also be demonstrated by the following examples:

Reacting N^{α}-acylated amino acid or peptide phenylhydrazides with N-bromosuccinimide, followed by treatment of the resulting diimides with anhydrous ethanol (6) produced the corresponding ethyl ester derivatives required for α-chymotrypsin-catalysis. For the same reason as described above identification of the products and reactants by position matching failed with respect to the following reactions:

Boc-Tyr-N_2H_2Ph ⟶ Boc-Tyr-OEt

Boc-Phe-N_2H_2Ph ⟶ Boc-Phe-OEt

Boc-Gly-Phe-N_2H_2Ph ⟶ Boc-Gly-Phe-OEt

Assignment of educts and products could be achieved according to procedures reported above for the α-chymo-

Table 1: Characterization of amino acid and peptide derivatives

Compound	R_F A[a]	R_F B[b]	Color reactions [d]	Color reactions [e]	Color with ninhydrin[c]	Elemental analyses	Found	Calcd.	Amino acid analyses	Found	Calcd.
Boc-Phe-N_2H_2Ph	0.85	0.85	+	−	reddish-brown (26)[f]	C	67.65	67.59			
						H	7.08	7.09			
						N	11.77	11.82			
Boc-Phe-OEt	0.87	0.89	−	−	violet (11)	C	65.54	65.51			
						H	7.80	7.90			
						N	4.51	4.77			
Boc-Phe-Leu-N_2H_2Ph	0.87	0.84	−	−	brown (23)	C	67.15	66.66			
						H	7.69	7.74	Leu	1.00	1.00
						N	12.06	11.96	Phe	0.96	1.00
Boc-Gly-Phe-N_2H_2Ph	0.84	0.68	+	−	yellowish-brown (22)	C	64.04	64.07			
						H	6.62	6.84	Gly	1.00	1.00
						N	13.73	13.59	Phe	1.02	1.00
Boc-Gly-Phe-OEt	0.85	0.85	−	−	reddish-brown (26)	C	61.86	61.71			
						H	7.18	7.48	Gly	1.00	1.00
						N	7.80	8.00	Phe	0.97	1.00
Boc-Gly-Phe-Leu-N_2H_2Ph	0.86	0.70	−	−	yellow (2)	C	64.40	63.98	Gly	1.00	1.00
						H	6.91	7.48	Leu	0.95	1.00
						N	13.42	13.32	Phe	0.98	1.00
Boc-Gly-Phe-Met-N_2H_2Ph	0.86	0.68	−	−	yellow (2)	C	59.84	59.56			
						H	6.93	6.86	Gly	1.00	1.00
						N	13.08	12.88	Met	0.94	1.00
						S	5.83	5.90	Phe	1.01	1.00
Boc-Gly-Leu-N_2H_2Ph	0.84	0.69	+	+	yellowish-orange (4)	C	60.23	60.30			
						H	7.96	7.99	Gly	1.00	1.00
						N	14.78	14.80	Leu	0.96	1.00

Boc-Tyr-N_2H_2Ph	0.84	0.67	+	+	brown (25)	C H N	64.32 6.84 11.52	64.64 6.78 11.36		
Boc-Tyr-OEt	0.84	0.77	—	—	reddish-violet (10)	C H N	61.75 7.39 4.49	62.13 7.49 4.53		
Boc-Tyr-Gly-N_2H_2Ph	0.77	0.49	+	+	orange (5)	C H N	61.43 6.49 13.16	61.68 6.59 13.08	Gly Tyr	1.00 0.91	1.00 1.00
Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt	0.89	0.70	—	—	reddish-violet (10)	C H N	65.68 7.00 8.52	65.44 6.71 8.48	Gly Tyr Phe	2.00 0.91 0.98	2.00 1.00 1.00
Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N_2H_2Ph	0.90	0.62	—	—	bluish-violet (12)	C H N	65.96 6.82 11.88	66.09 6.87 11.73	Gly Leu Tyr Phe	2.00 0.94 0.89 0.95	2.00 1.00 1.00 1.00

[a]Solvent system A: chloroform-methanol(3:1); [b]Solvent system B: chloroform-methanol-acetic acid(45:4:1); [c]Colors have been determined 30 min after ninhydrin treatment by use of a hue indication chart; [d]Reaction with Sakaguchi-reagent; [e]Reaction with sodium nitroprusside; [f]Arabic numerals given in brackets refer to hue differences illustrated in the chart, which forms part of a color index (7).

trypsin-mediated syntheses. The results are depicted in Table 1.

As the outcome of protease-catalyzed peptide syntheses is sometimes unpredictable the nature of the resulting products requires critical evaluation. This may be explained by the following reaction: the tripeptide Boc-Gly-Phe-Leu-N_2H_2Ph was an essential intermediate in the preparation of Leu-enkephalin(2). It could be synthetized via α-chymotrypsin-catalysis (vide supra). Nevertheless, it was attempted to prepare it by papain-mediated coupling of Boc-Gly-Phe-OH and H-Leu-N_2H_2Ph as well. Monitoring of the papain-catalyzed reaction by means of thin-layer chromatography using the solvent systems A and B revealed a ninhydrin-positive spot, which had moved to a position hardly distinguishable in both systems from that occupied by the abovementioned tripeptide. Spraying with Sakaguchi-reagent and sodium nitroprusside indicated the presence of a phenylhydrazide derivative. However, visual comparison of the two spots after ninhydrin-treatment suggested non-identity of the two products (Table 1). This assumption could be confirmed by both amino acid and elemental analysis, which proved the substance in question to be the dipeptide Boc-Gly-Leu-N_2H_2Ph (Table 1). Obviously papain had at the very beginning hydrolyzed the dipeptide Boc-Gly-Phe-OH to furnish H-Phe-OH, which could be

It is taken for granted, that the reaction of amino acids and peptides with ninhydrin proceeds via Schiff bases (8,9) an integral part of which are the distinct amino acid side chains. As the formation of differing Schiff bases is responsible for the varied color developments (8), the divers color reactions of Boc-Gly-Leu-N_2H_2Ph and Boc-Gly-Phe-Leu-N_2H_2Ph on the one hand and of Boc-Tyr-Gly-N_2H_2Ph and Boc-Tyr-N_2H_2Ph on the other hand seems explicable if one assumes that they are influenced not only by the first but also by the second amino acid side chain of the peptides.

The Sakaguchi-reagent is commonly used in peptide chemistry to identify arginine residues (10). During this work it proved also to be a suitable agent to detect phenylhydrazide protected amino acids and peptides. The color reaction presumably implies the oxidation by sodium hypobromite of the phenylhydrazide moiety to give the corresponding phenyldiimide group, which is subsequently removed upon reaction with 1-naphthol to form 4-phenylazo-1-naphthol.

The preliminary characterization of each of the compounds described above could be verified by elemental analysis. As far as peptides were concerned amino acid compositions were determined as well (Table 1).

traced by thin-layer chromatography, and Boc-Gly-OH, which subsequently reacted with H-Leu-N_2H_2Ph via papain-catalysis to give Boc-Gly-Leu-N_2H_2Ph.

The attempt to synthetize Boc-Tyr-Gly-N_2H_2Ph by papain-mediated reaction between Boc-Tyr-OH and H-Gly-N_2H_2Ph resulted in a product, which could be shown to be a phenylhydrazide compound according to hypobromite - 1-naphthol and sodium nitroprusside treatment. Notwithstanding these results, the corresponding R_F-values provided strong evidence, that the product in question was not identical with the desired dipeptide. Color matching using ninhydrin suggested the compound to be Boc-Tyr-N_2H_2Ph. This characterization could be confirmed by elemental analysis (Table 1).

The varied colors observed for ethyl esters and the corresponding phenylhydrazide derivatives may be explained in terms of the oxidizing power of ninhydrin, which presumably transforms the latter into their phenyldiimide analogs. These azo compounds displayed a faintly yellow color on thin-layer plates already in the absence of ninhydrin. The above reasoning is supported by the finding that a substance resulting from ninhydrin-treatment of H-Phe-N_2H_2Ph (20 min, 110°C) exhibited the same behavior as phenylalanine diimide with regard to both color and position when run in the solvent systems 1-butanol-pyridine-acetic acetic-water (20:10:3:10) and chloroform-methanol (3:1).

REFERENCES

1 Kullmann, W., Proteases as catalysts for enzymic syntheses of opioid peptides, J. Biol. Chem., 255, 8234, 1980

2 Kullmann, W., Protease-mediated peptide bond formation. Some unexpected outcomes during enzymatic synthesis of Leu-enkephalin, J. Biol. Chem., 256, 1301, 1981

3 Wünsch, E., in 'Methoden der organischen Chemie' Synthese von Peptiden (ed. E. Müller) Vol. 15, Part 2 (Houben-Weyl) Thieme, Stuttgart, 1974

4 Morihara, K., and Oka, T., α-Chymotrypsin as the catalyst for peptide synthesis, Biochem.J. 163, 531, 1977

5 The abbreviations used are: Boc, t-butyloxycarbonyl; Bzl, benzyl; Ph, phenyl; OEt, ethyl ester.

6 Milne, H.B. and Kilday, W., Peptide synthesis of N-acetyl-α-amino acid phenylhydrazides, J. Org. Chem., 30, 64, 1965.

7 Colour Index, 3rd ed., Vol.I, (ed.) Society of Dyers and Colourists, Bradford, Yorkshire, 1971.

8 Mc Caldin, D.C., The chemistry of ninhydrin, Chem. Rev. 60, 39, 1960

9 Larsen, B.R., Lazzari, E.P., and Desiderio, D.M., Products of the reaction of ninhydrin with triphenylalanine, Anal. Biochem. 86, 127, 1978.

10 Stahl, E., Dünnschichtchromatographie, 2nd ed., Springer-Verlag, Berlin, 1967.