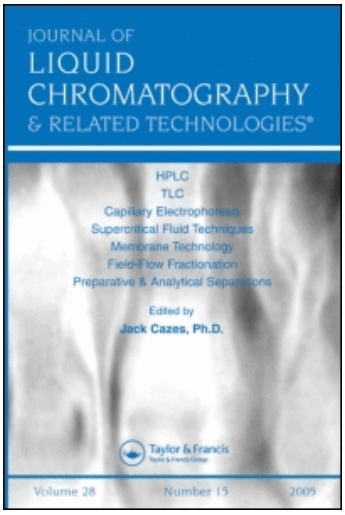

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P. Slonina[a]; E. Krause[b]; A. Hänsicke[a]
[a] Berlin-Chemie, Berlin, AG, Germany [b] Institute of Drug Research, Berlin, Germany

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LARGE SCALE PURIFICATION OF IMMUNREGULATORY PEPTIDE $N^{\alpha 1}$, $N^{\epsilon 2}$-DIACETYL-SPLENOPENTIN BY CHROMATOGRAPHY ON A CARBOXYLATED CATION EXCHANGER

P. SLONINA[1], E. KRAUSE[2], AND A. HÄNSICKE[1]
[1]*Berlin-Chemie AG*
0-1199 Berlin, Germany
[2]*Institute of Drug Research*
0-1136 Berlin, Germany

ABSTRACT

The immunregulatory pentapeptide ARG-LYS-GLU-VAL-TYR was synthesized by using the mixed anhydride coupling method. Acylation of $N^{\alpha 1}$- and $N^{\epsilon 2}$-amino groups increases the enzymatic degradation stability of splenopentin. This paper describes a simple and effective large scale purification method of $N^{\alpha 1}$-, $N^{\epsilon 2}$-diacetyl-splenopentin synthesis product by chromatography on the carboxylated cation exchanger Wofatit Y 79. Using ethanol/acidic water as eluent the isolation of purified peptide from the eluat was achieved directly by evaporation under vacuum and lyophilisation.

INTRODUCTION

As the result from extensive studies on the extraction and characterisation of immunbiologically active peptides in the last years thymopoietin and splenin were isolated from thymus gland and spleen respectively. Despite of different organ origin they show a wide structure similarity. Thymopoietin affect neuromuscular transmission and induce the phenotypic differentiation of T precursor cells while inhibiting phenotypic differentiation of B

cells. Splenin, in contrast, does not affect neuromuscular transmission and induces T- and B-cell precursors /1/.

The biological potency of splenin is retained by its sequence section 32-36 Arg-Lys-Glu-Val-Tyr (splenopentin). Acylation of the splenopentin causes an increase of the enzymatic degradation stability resulting in a prolonged efficacy /2/. The synthesis of $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin was performed by step wise formation of the peptide chain from C-term using the mixed anhydride coupling method, hydrogenolytic deprotection and acylation by AcONB /2/.

Chromatographic purifications of peptides are usually carried out by adsorption or distribution chromatography on silica, ion exchange chromatography and/or preparative reversed-phase HPLC /3-5/. Most of the ion exchange operations are characterized by the application of salt containing elution systems requiring an additional desalting step. Concerning preparative HPLC the high costs for RP-silica compared with ion exchange materials should be considered as an important economical parameter in large scale chromatography. In order to perform adsorption or distribution chromatography on silica, large amounts of different organic solvents are necessary. The high purity of these solvents and its continous redestillation must be secured.

The submitted paper describes a simple method for purification of $N^{\alpha 1}$-, $N^{\epsilon 2}$-diacetyl-splenopentin synthesis product by large scale chromatography on a carboxylated cation exchanger.

MATERIALS AND METHODS

Analytical HPLC

Analytical HPLC separations of the peptides were carried out on LiChrosorb RP 18 (250 x 4 mm I.D.) 7 µm. The instrumentation consisted of a HPLC pump 64 (KNAUER, FRG), Rheodyne Model 7125 injection valve, a variable-wavelength UV detector 87 (KNAUER, FRG), operating at 220 nm.

Eluent A 13% acetonitrile/ 87% 0.05 M KH_2PO_4, pH 3,0; Eluent B 20% acetonitrile/ 80% 0,05 M KH_2PO_4, pH 3,0.

Separations were performed at ambient temperature and at an eluent flow-rate of 0,5 ml/min.

HPLC grade acetonitrile was purchased from PCK Schwedt, FRG.

Preparation of the ion exchanger

Carboxylated cation exchanger Wofatit Y 79, particle size 50-100 µm, was purchased from Bitterfeld AG, FRG.
The resin (7 kg) was prepared for preparative separation by equilibration with 140 l 1 N NaOH followed by washing with water until the pH was 7. Equilibration with 140 l 1 N HCl converts the ion exchanger into the H-form. After washing with water (pH 4) the resin was suspended with 40 l 37,5% ethanol/ 3 x 10^{-4} N HCl and filled into the column. Equilibration of the column was performed with 100 l 37,5% ethanol/ 3 x 10^{-4} N HCl.
Columns dimensions 1000 mm x 200 mm I.D., bed height 640 mm, bed volume 20,1 l.
All solutions were made with deionized water. NaOH, HCl, Acetic acid and ethanol had p.A. grade quality.

Peptides

$N^{\alpha 1}$-,$N^{\epsilon 2}$-Diacetyl-splenopentin and all reference substances were synthesized by stepwise formation of the peptide chain from the C-term using the mixed anhydride coupling method /2/.

RESULTS AND DISCUSSION

Analysis of the synthesis product

The $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin synthesis product was analyzed by reversed-phase HPLC to determine the contents of $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin and the peptide impurities (Fig. 1). Two isocratic elution systems containing 13 % and 20 % acetonitrile were used, respectively. The impurities could be identified by chromatography of reference substances. The results are summerized in table 1.

Ion exchange chromatography on Wofatit Y 79

Besides dominating ionic forces it was found that van der Waals and hydrophobic interactions seem to play an important role at the adsorption process on carboxylated cation exchanger Wofatit Y 79 . The balance of both forces can be changed systematically varying pH, ionic strength or solvent concentration in the mobile phase.

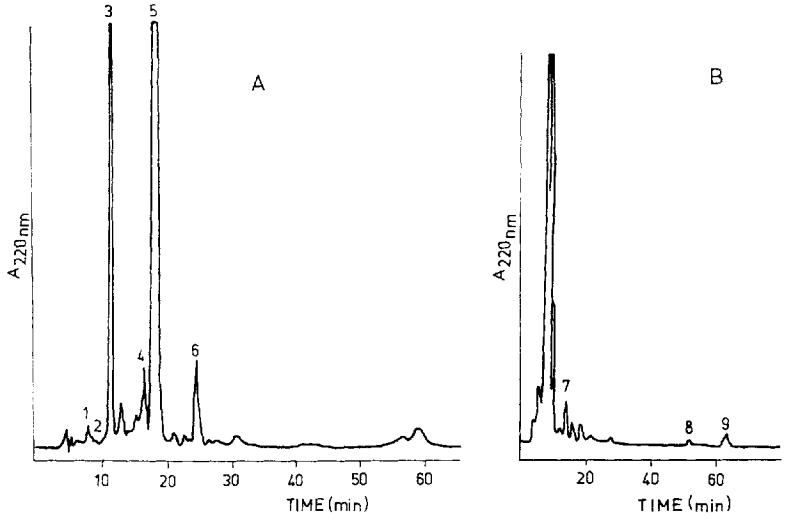

Figure 1

Analytical reversed-phase HPLC of $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin synthesis product on a Lichrosorb RP 18 column (250 x 4 mm I.D.), 7 µm
Eluent A: 13 % Acetonitril / 87 % 0,05 M KH_2PO_4, pH 3,0 (v/v)
Eluent B: 20 % Acetonitril / 80 % 0,05 M KH_2PO_4, pH 3,0 (v/v)

TABLE 1

Peak No.	Substance
1	$N^{\alpha 1}$-Monoacetyl-splenopentin
2	$N^{\epsilon 2}$-Monoacetyl-splenopentin
3	HONB
4	$N^{\alpha 1}$-,$N^{\epsilon 2}$-,N^{w1}-Triacetyl-splenopentin
5	$N^{\alpha 1}$-,$N^{\epsilon 2}$-Diacetyl-splenopentin
6	$N^{\alpha 2}$-,$N^{\epsilon 2}$-Diacetyl-(des-Arg^1)-splenopentin
	$N^{\alpha 1}$-,$N^{\epsilon 2}$-Diacetyl-[D-Tyr^5]-splenopentin
7	$N^{\alpha 1}$-,O^{w5}-Diacetyl-splenopentin
8	$N^{\alpha 2}$-Boc-,$N^{\epsilon 2}$-Monoacetyl-(des-Arg^1)-splenopentin
9	$N^{\alpha 2}$-i-Boc-,$N^{\epsilon 2}$-Monoacetyl-(des-Arg^1)-splenopentin

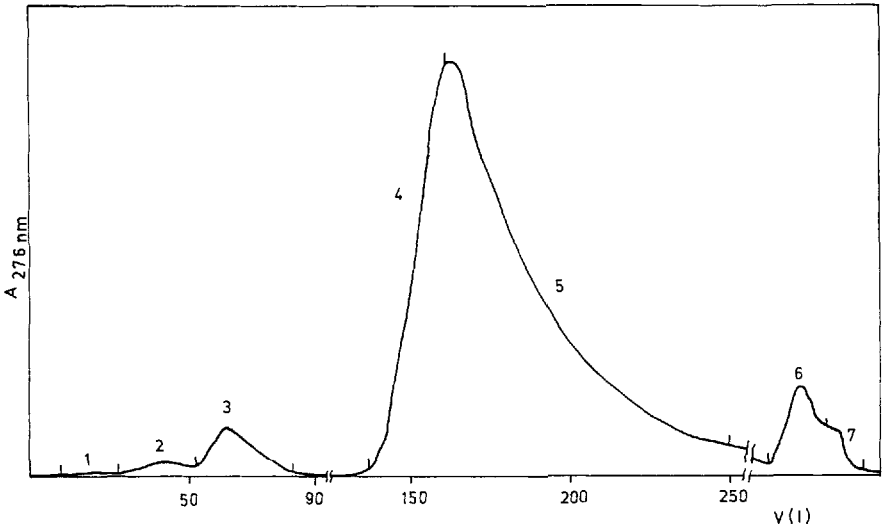

Figure 2

Elution of 200 g $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin synthesis product on the Wofatit Y 79 column (640 x 200 mm I.D) in 37,5 % ethanol, 3 x 10^{-4} N HCl with a flow rate of 10 l/h

Separating positively charged solutes, the influence of the carboxyl groups of the ion exchanger (pK 4,7) is decreased either by lowering pH or increasing salt concentration in the eluent The application of organic solvents at ion exchange processes can improve the solubility of peptides , but they also influence adsorption of the solute at the surface by different interactions (decrease of solvatation, change of pK values of the functional groups, electrostatic interaction). Retention of both charged and uncharged solutes on the carboxylated cation exchanger strongly depends on the eluotropic strength (methanol < ethanol < acetonitrile) and the concentration of the solvent applied. However , uncharged substances are commonly less retained on Wofatit Y 79 than charged solutes provided that the adsorbent is hold in the ion exchange mode. This directly correlates with the different strengths of adsorption and ion exchange energies /6/.

Chromatographic procedure

200 g $N^{\alpha 1}$-,$N^{\epsilon 2}$-Diacetyl-splenopentin was solved in 10 l 3 x 10^{-4} N HCl and loaded onto the Wofatit Y 79 column. After washing with

TABLE 2

IDENTIFICATION OF THE PREPARATIVE ION EXCHANGE FRACTIONS

Fraction	Substances
1	Breakthrough, unidentified mixture (< 1 %)
2	HONB
3	$N^{\alpha 2}$-,$N^{\epsilon 2}$-Diacetyl-(des-Arg^1)-splenopentin
	$N^{\alpha 2}$-Boc-,$N^{\epsilon 2}$-Monoacetyl-(des-Arg^1)-splenopentin
	$N^{\alpha 2}$-i-Boc-,$N^{\epsilon 2}$-Monoacetyl-(des-Arg^1)-splenopentin
4	$N^{\alpha 1}$-,$N^{\epsilon 2}$-,N^{w1}-Triacetyl-splenopentin (6 %)
	$N^{\alpha 1}$-,$N^{\epsilon 2}$-Diacetyl-splenopentin
5	$N^{\alpha 1}$-,$N^{\epsilon 2}$-Diacetyl-splenopentin (HPLC Fig. 3)
6	$N^{\alpha 1}$-,O^{w5}-Diacetyl-splenopentin
7	$N^{\alpha 1}$-Monoacetyl-splenopentin
	$N^{\epsilon 2}$-Monoacetyl-splenopentin

two bed volumes 3 x 10^{-4} N HCl (fraction 2) the first elution process with 37,5 % ethanol /3 x 10^{-4} N HCl (pH 3,5) was started (fraction 3-5). After desorption of the main product second elution with 50 % ethanol /1 N Acetic acid completed the process (fraction 6-7). The elution diagram of the purification is shown in Fig. 2.

Peptides in the collected fractions were identified by reversed-phase HPLC using reference substances (Table 2).

It is apparent that uncharged substances almost completely break trough (fraction 3). The elution behaviour of $N^{\alpha 1}$-,$N^{\epsilon 2}$-,N^{w1}-triacetyl-splenopentin is not very clear. Although it has not any free charge it coelutes with $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin (one charge) in the same order as it was observed on reversed-phase HPLC conditions. Substances with two positve charges (monoacetyl-splenopentins, $N^{\alpha 1}$-,O^{w5}-diacetyl-splenopentin) that differ from $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin by great lipophilicity show the highest retention.

Isolation of purified $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin

The volume of fraction 5 was reduced to 5 l by evaporation under vacuum. After lyophilisation the average substance yield runs to

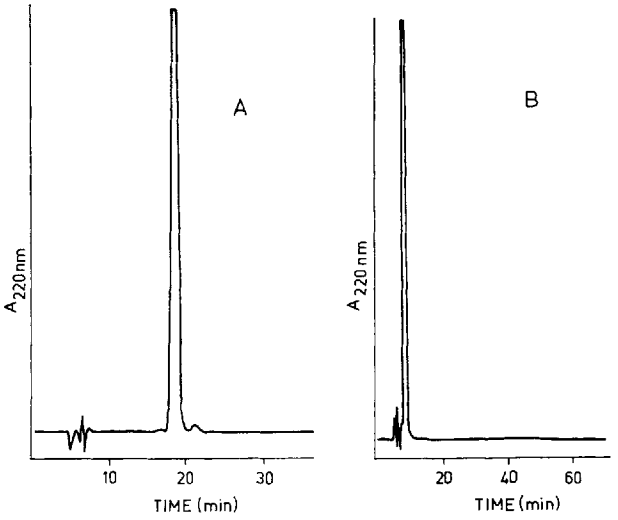

Figure 3

Analytical reversed-phase HPLC of purified $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin on a Lichrosorb RP 18 column (250 x 4 mm I.D.), 7 µm
Eluent A: 13 % Acetonitril / 87 % 0,05 M KH_2PO_4, pH 3,0 (v/v)
Eluent B: 20 % Acetonitril / 80 % 0,05 M KH_2PO_4, pH 3,0 (v/v)

50 % related to the amount of applied synthesis product.

The purity of the isolated peptide was determined by reversed-phase HPLC with two mobile phases to be 99,0 % with eluent A and 98,2 % with eluent B, respectively (Fig. 3).

Substance recovery over the whole chromatography process was found to be > 95 %.

Fraction 4 was collected and rechromatographed. The Wofatit Y 79 resin is to be regenerated after 3 chromatography runs.

CONCLUSIONS

These results demonstrate a simple and effective procedure to purify $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin synthesis product by large scale chromatography on the carboxylated cation exchanger Wofatit Y 79.

The advantages of this method can be summarized as follows. The chromatography is performed in a solvent/aqueous acid mixture. Ethanol acts both as organic modifier and desolvating agent. It apparently causes a decrease of ionic interaction and substitutes commonly used salt containing eluents. An additional desalting step is avoided. The direct isolation of pure peptide from solution by evaporation and lyophilisation becomes possible. Despite of the simple chromatographic procedure the final product obtained had 98 % purity by reversed-phase HPLC. An average yield of 100 g purified $N^{\alpha 1}$-,$N^{\epsilon 2}$-diacetyl-splenopentin from 200 g synthesis product is outlined.

It should be mentioned that the described method was applied succesfully for the chromatographic purification of insulin /7/ and substance P using some altered conditiones.

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