Journal of Chromatography, 346 (1985) 271–279
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 941

ISOTACHOPHORETIC CONTROL OF PEPTIDE SYNTHESIS AND PURIFICATION

A NOVEL APPROACH USING ULTRAVIOLET DETECTION AT 206 nm

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(Received June 3rd, 1985)

SUMMARY

The synthesis and purification of two glutamine-containing dipeptides were controlled by applying analytical capillary isotachophoresis. The use of a newly developed detector block allowed conductivity detection and, for the first time, UV measurement at 206 nm. This system facilitates qualitative and quantitative analysis of peptides not absorbing at 254 and 280 nm in amounts of less than 200 ng, thereby permitting a direct classification of the sample ions analysed. Optimization of peptide synthesis is significantly improved by this novel method, which enables simultaneous monitoring of the reaction products and contaminating inorganic ions during synthesis and purification.

INTRODUCTION

In a previous communication, synthesis of peptides containing glutamine (Gln) and tyrosine (Tyr) was described[1], and in subsequent reports the potential use of analytical capillary isotachophoresis in characterizing these synthetic products was emphasized[2,3]. In the present study, the synthesis and purification of two Gln-containing dipeptides, L-alanyl-L-glutamine (Ala-Gln) and L-aspartyl-L-glutamine (Asp-Gln) were isotachophoretically controlled using conductivity and UV detection. A newly developed detector block allowed measurements of the UV signal not only at the commonly used wavelengths (254 and 280 nm), but also at 206 nm, which is especially suited for peptide bonds. This novel method of detection enabled satisfactory analysis of peptide material in amounts less than 200 ng, which is not feasible with the commonly used detector systems. The use of the proposed detector block facilitates detection of peptides not absorbing at 254 and 280 nm. Thus, this new qualitative and quantitative approach may represent a novel method for controlling peptide synthesis and purification procedures.

0021-9673/85/$03.30

EXPERIMENTAL

Peptide synthesis and purification

Ala-Gln and Asp-Gln were synthesized by the N-carboxy anhydride (NCA) method described previously[1,4]. The synthesized peptides were purified by repeated gel filtration on Sephadex G-10 (Pharmacia, Uppsala, Sweden)[1]. Crude materials, intermediates and end products were isotachophoretically analysed in concentrations and amounts given in the figures.

Analytical isotachophoresis

Analytical isotachophoresis was performed by using a LKB 2127 Tachophor equipped with an automatic driving current control unit. Separations were made in a PTFE capillary (230 × 0.55 mm I.D.) attached to a newly developed detector block (LKB 2127-140), allowing detection of the linear conductivity signal and UV absorption at 254/280 nm and 206 nm. Mercury (254 nm) and iodine (206 nm) were used as light sources. The detection signals were monitored by employing a two-channel recorder (Kipp & Zonen, Delft, The Netherlands) with a chart speed of 6 cm/min. The separations required *ca.* 10 min and the detection current was 60 μA.

The operational systems used are outlined in Table I. These solutions were prepared from analytical grade chemicals provided by E. Merck (Darmstadt, F.R.G.) and Sigma (St. Louis, MO, U.S.A.) as previously described[2,3].

As reference, aqueous solutions of alanine (Ala) and aspartic acid (Asp) (E. Merck) as well as glutamine (Gln) and L-alanyl-L-alanine (Ala-Ala) (Sigma) were investigated alone or in doping experiments.

RESULTS

Control of peptide synthesis

Three crude specimens of Ala-Gln derived under various synthesis conditions were analysed with electrolyte system 1 (Table I). The respective isotachopherograms are shown in Fig. 1. Using conductivity detection and UV measurement at 254 nm (Fig. 1A) analyses of the crude products revealed at least five non-UV-absorbing

TABLE I

OPERATIONAL SYSTEMS FOR ANIONIC ANALYSIS

MES = Morpholinoethanesulphonic acid; ammediol = 2-amino-2-methyl-1,3-propanediol; HPMC = hydroxypropylmethylcellulose.

	Leading electrolyte	*Terminating electrolyte*
System 1	0.005 *M* MES Ammediol 0.4% HPMC pH 9.1	0.01 *M* β-alanine Ammediol $Ba(OH)_2$ pH ≈10
System 2	0.01 *M* Cl^- Bis-Tris 0.4% HPMC pH 6.0	0.01 *M* MES Tris pH ≈6

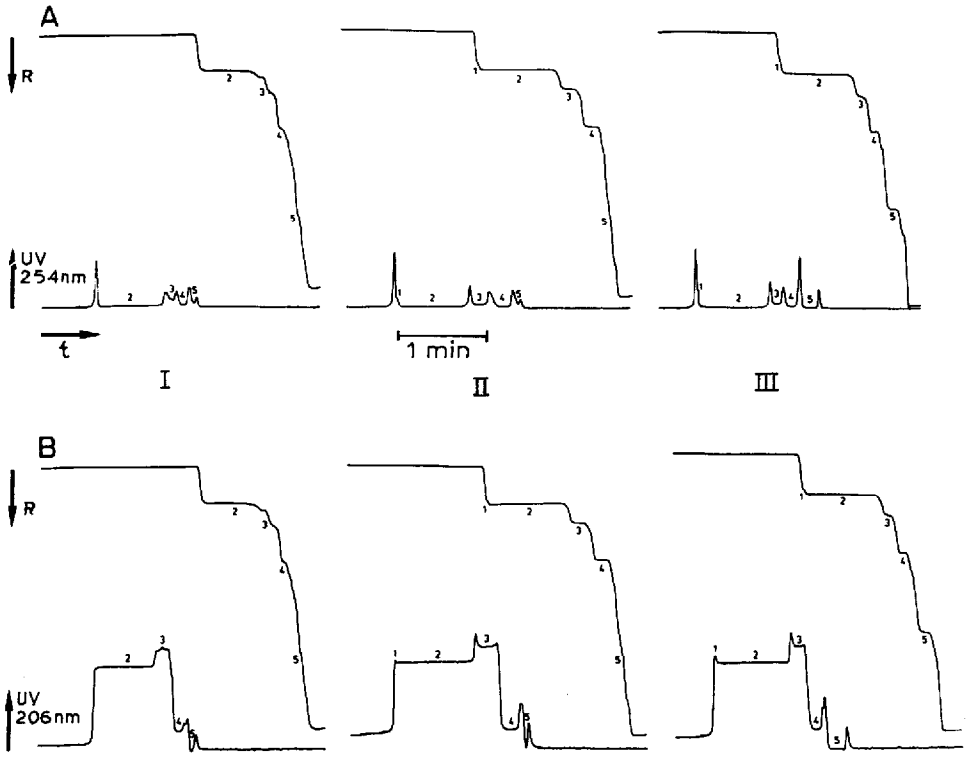

Fig. 1. Isotachophoretic analyses (electrolyte system 1) of three different crude samples of Ala-Gln (I–III): UV detection at 254 nm (A) and 206 nm (B). For each separation, 20 μl were injected, corresponding to 8.68 μg of material. Key: 1 = Ala-Ala; 2 = Ala-Gln; 3 = unknown peptide(s); 4 = Gln; 5 = Ala.

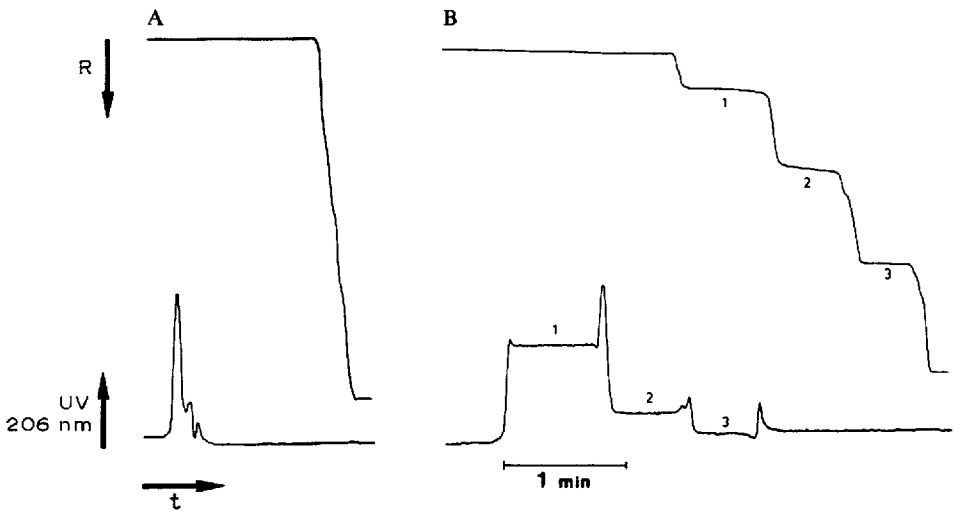

Fig. 2. Isotachophoretic analysis (electrolyte system 1) of a reference mixture of Ala-Ala (1), Gln (2) and Ala (3). (A) electrolyte system; (B) reference mixture (2.5 μl injected, corresponding to 0.80 μg of Ala-Ala, 0.73 μg of Gln and 0.45 μg of Ala).

zones, as indicated by the conductivity signal. Four of these zones exhibited characteristic UV levels with a detection wavelength of 206 nm (Fig. 1B). As shown, the main zone as well as two minor zones revealed high UV absorption, obviously suggesting that they correspond to peptide material. One other minor zone, showing a lower UV absorption, may represent unchanged starting material. These assumptions were verified by analysing reference substances (Fig. 2). Compared with the isotachophoretic pattern of electrolyte system 1 (A), analysis of an equimolar standard mixture (B) resulted in two UV-absorbing zones and one non-UV-absorbing zone, corresponding to the dipeptide Ala-Ala and the free amino acids Gln and Ala.

Analyses of three different crude samples of Asp-Gln were performed by applying electrolyte system 2 (Table I) showing five discrete separated zones in various amounts (Fig. 3). None of these compounds exhibited UV absorption at 254 nm (Fig. 3A), whereas characteristic UV levels compared with the leading ion were measured at 206 nm (Fig. 3B). The main synthesis product, as well as three minor unidentified side products, showed high UV absorption, suggesting the presence of peptide bond(s) in these compounds, whereas the fifth zone exhibited low UV absorption. This latter zone was identified as free Asp by analysing the reference substance. The isotachopherograms of the electrolyte system alone (A) and of a standard solution of Asp (B) are depicted in Fig. 4.

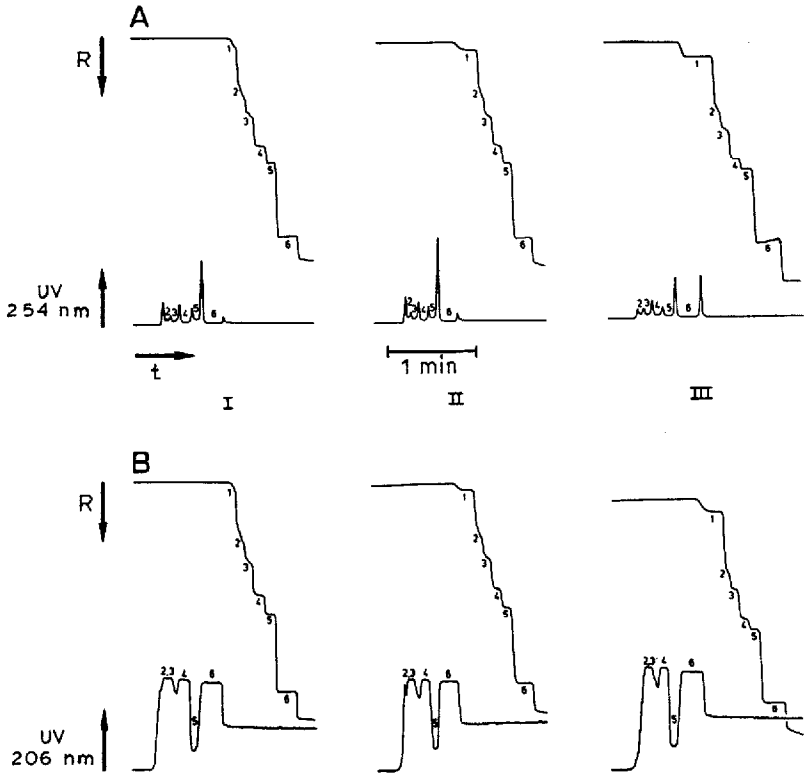

Fig. 3. Isotachophoretic analyses (electrolyte system 2) of three different crude samples of Asp-Gln (I–III): UV detection at 254 nm (A) and 206 nm (B). For each separation, 10 μl were injected, corresponding to 5.49 μg of material. Key: 1 = sulphate; 2, 3, 4 = unknown peptides; 5 = Asp; 6 = Asp-Gln.

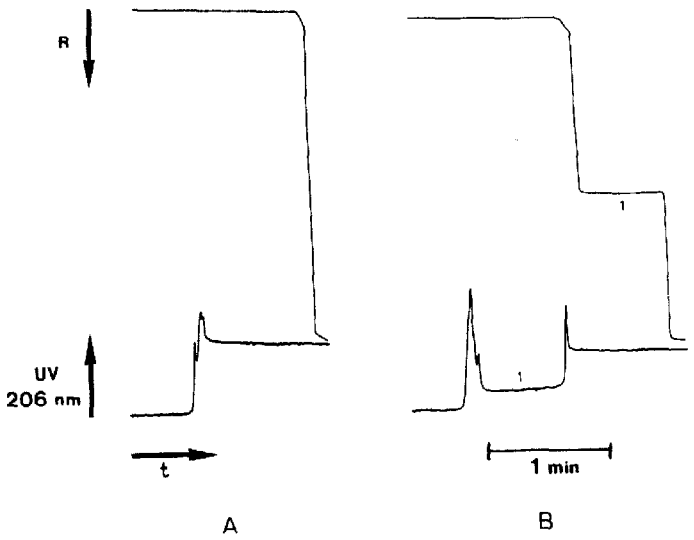

Fig. 4. Isotachophoretic analysis (electrolyte system 2) of a reference solution of Asp (1). (A) electrolyte system; (B) reference solution (10 μl injected, corresponding to 2.66 μg of Asp).

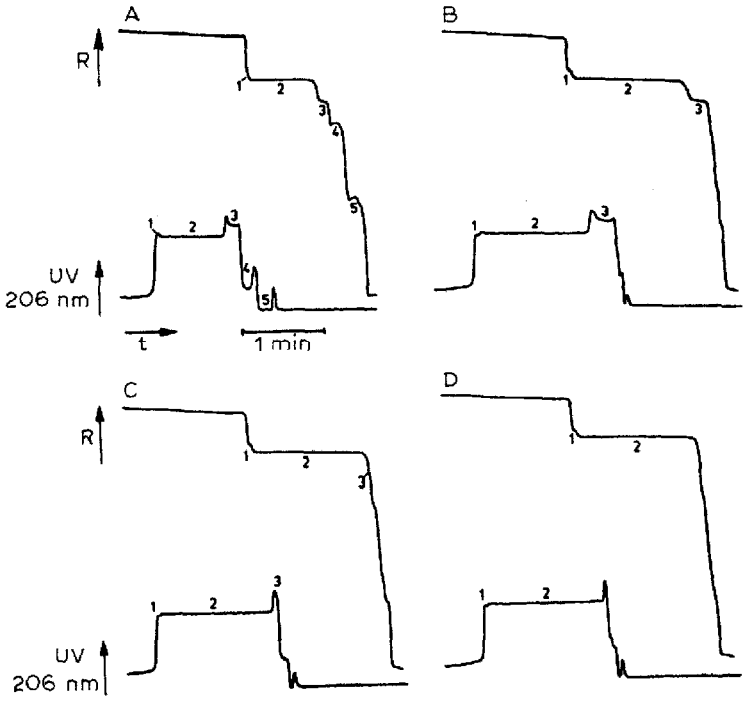

Fig. 5. Isotachophoretic analyses (electrolyte system 1) of Ala-Gln before (A) and after (B–D) the different purification steps. Key as in Fig. 1. For each separation 20 μl were injected, corresponding to 8.68 μg of material.

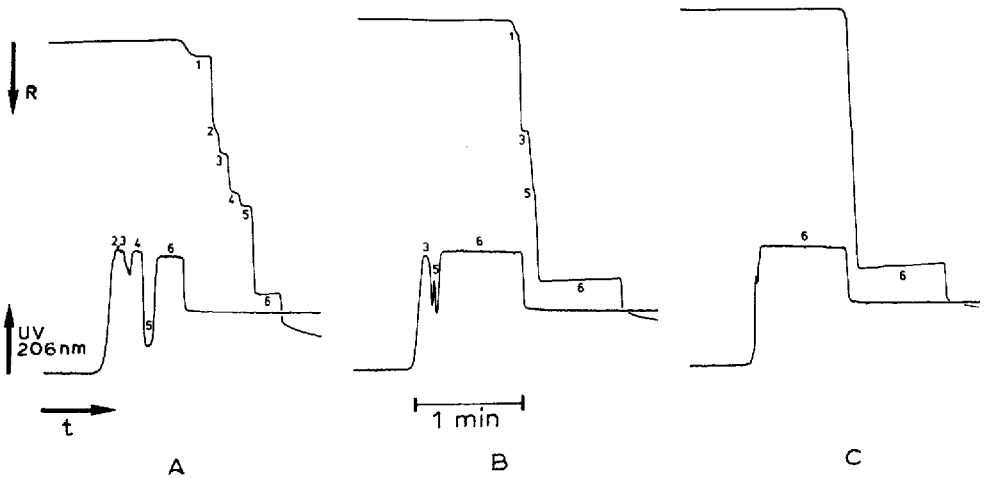

Fig. 6. Isotachophoretic analyses (electrolyte system 2) of Asp-Gln before (A) and after (B,C) the different purification steps. Key as in Fig. 3. For each separation 10 μl were injected, corresponding to 5.49 μg of material.

In all Asp-Gln fractions analysed a further zone, only detectable in the conductivity signal, was observed. This suggested the presence of contaminating sulphate derived from the peptide synthesis procedure.

Control of purification process

The crude material and the different peptide fractions obtained during the development of purification of Ala-Gln were analysed by conductivity detection and UV measurement at 206 nm. The respective isotachopherograms are shown in Fig. 5. Compared with the analysis of the crude material (A), both free amino acids Ala and Gln could be removed during the first gel-chromatographic purification step (B). Furthermore, the zone length for the main product was markedly increased, and moderate increases were also noted for Ala-Ala and the unidentified side product. This might be due to simultaneous removal of salts during the first purification step. The majority of the two contaminating products could be removed by applying further gel filtration purification steps (C,D). In the final product an enrichment of Ala-Gln of approaching 100% could be achieved. Only traces of Ala-Ala were detectable.

The crude material (A) and the different purified peptide fractions (B,C) of Asp-Gln were analysed similarly but with electrolyte system 2 (Table I), as illustrated in Fig. 6. No free Asp and only small amounts of contaminating peptides could be detected after the first purification step. After the second gel-chromatographic separation an enrichment of approaching 100% was observed for Asp-Gln.

DISCUSSION

Although in several investigations analytical capillary isotachophoresis was found to be a promising tool in qualitative and quantitative analyses of synthetic[2,35–9]

and naturally occuring[10–16] peptides, only a few investigators have applied this method as a control in peptide purification process[17–20].

Kopwillem *et al.* analysed the crude material as well as peptide-containing fractions obtained during the purification of synthetic tetradeca-, deca- and undecapeptides[17–19]. Friedel and Holloway employed analytical isotachophoresis in the analysis of intermediates and end products during synthesis of a pentapeptide[20]. In these studies detection was made by monitoring thermometric and UV signals. Owing to the relatively low sensitivity of thermometric detection, high amounts (*ca.* 30 μg) of peptide material were necessary to obtain satisfactory results. Additionally, the characteristic wave form of the thermometric signal rendered a quantitative and qualitative evaluation difficult. Thus, only the enrichment of the main products during the purification process could be followed. In these studies the UV absorption was monitored at 254 nm and/or 280 nm, so only peptides containing aromatic amino acids and cystine revealed characteristic UV absorption.

In general, s.c. "marker or separation peaks" (presumably due to different refractive indexes of the separated zones) mark the respective zone boundaries of non-UV-absorbing solutes allowing evaluation of the UV signal. Nevertheless, in certain operational systems no such "marker peaks" are detectable, thereby invalidating evaluation of the UV signal[21,22]. Furthermore, lack of characteristic UV levels at 254/280 nm complicates the identification and classification of unknown sample compounds.

Up to date, measurement of UV absorption at lower wavelengths has not been feasible, since in all commercially available built-in UV detectors the signal was measured directly through the capillary wall. Thus, the high absorption and dispersion of the capillary material (generally PTFE) hindered the use of wavelengths below 230 nm. In the present study, a new commercially available detector block was applied, which allowed concomitant conductivity and UV measurements. The great improvement of the new UV detector lies in the fact that the fibre optics lead the light directly into the interior of the liquid system. This results in a significantly lower loss of UV light intensity compared with the previous types of capillary plate. Consequently, this new detector block facilitates UV measurement at 206 nm.

A limiting factor in using UV detection at 206 nm is the considerably increased absorption of the buffer solutions at this wavelength. As demonstrated by Verheggen *et al.*[23], operational systems at alkaline pH including chloride or MES as leading and Ammediol or Tris as counter ion show low absorption at 206 nm. In contrast, anionic operational systems at pH 6 with histidine as counter ion are not suitable, owing to the strong absorption of this amino acid at lower wavelengths[23]. Therefore, preparing an operational system at pH 6, we replaced histidine with Bis-Tris (pK_a 6.5) which showed a high buffer capacity at this pH value and low UV absorption at 206 nm (Figs. 3, 4 and 6).

The advantage of measuring UV absorption at 206 nm compared with 254 nm is illustrated in Figs. 1 and 3, which show that analyses of both Gln-containing peptides at 254 nm revealed several non-UV-absorbing zones. Since no characteristic UV levels could be measured at this wavelength, no information about the chemical structure of the separated compounds could be obtained. Consequently, for further characterization of sample ions suitable reference substances are required. In contrast, UV detection at 206 nm facilitates a direct classification of the sample ions,

enabling a direct differentiation between peptides formed during synthesis and unchanged starting materials (Figs. 1 and 3). This is of great importance in the optimization of peptide synthesis by the modification of reaction variables: pH, temperature and especially starting amino acid material.

According to the high absorption coefficient of peptide bonds at 206 nm[24], all zones with high UV absorption must correspond to peptides, whereas all zones with lower or no UV absorption could be unreacted free amino acids, as verified when analysing the respective standard mixtures shown in Figs. 2 and 4, respectively. Indeed, it is evident that the characteristic conductivity step heights and UV levels derived on the one hand from the crude preparations and on the other hand from the standard substances are identical. The main Gln-containing products could be identified and characterized after purification, as previously described[1].

Apart from the qualitative approach, isotachophoresis provides quantitative information about the composition of the samples analysed[2,3,12,13,21]. In this context one should remember that the concentrations of the zones are directly related to the concentration of the leading ion[21]. Thus, the ratio of the main peptide formed to remaining starting material and side products can be estimated by measuring single zone lengths and total zone length, respectively. Calculation of this ratio may serve as a guideline to define optimum conditions for synthesis (Figs. 1 and 3) and purification (Figs. 5 and 6).

This quantitative approach underlines the obvious advantage of isotachophoresis compared with high-performance liquid chromatography (HPLC), which requires the use of suitable reference substances for a proper quantitative evaluation of analysed material. Indeed, the major advantages of isotachophoresis over HPLC lie in the facts that no processing of samples is necessary prior to analysis[9] and that simultaneous detection of contaminating ions is feasible[25]. This latter advantage is emphasized in Figs. 3 and 6, which show the presence of contaminating sulphate acquired from sulphuric acid in the reaction mixture used for peptide synthesis.

In the present study, the simultaneous use of unspecific conductivity detection and UV monitoring at 206 nm obviously increased the resolution and sensitivity compared with previously used detection systems, enabling quantitative measurement of peptide material in amounts of less than 200 ng. Thus, the introduction of the new detector block and its subsequent application might ameliorate the control of synthesis and purification procedures in peptide chemistry.

ACKNOWLEDGEMENTS

This work was supported by Arbeitsgemeinschaft Industrieller Forschungsvereinigungen (AIF, Nr. 5986). The kind support from Pfrimmer and Co. (Erlangen, F.R.G.) is gratefully acknowledged.

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