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ANALYTICAL CONTROL OF ENZYME-CATALYZED PEPTIDE SYNTHESIS USING CAPILLARY ISOTACHOPHORESIS

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SUMMARY

Analytical capillary isotachophoresis (ITP) was employed to control an enzyme-catalyzed peptide synthesis and the subsequent purification, as well as to evaluate the amino acid composition after acid and enzymatic hydrolysis. Compared with alternative chromatographic techniques, ITP offers certain advantages such as simultaneous detection of the synthetic peptide, amino acids, amino acid derivatives and contaminating inorganic ions in amounts of less than 200 ng. In addition, ITP provides quantitative information about the composition of the untreated samples directly analyzed without the mandatory use of suitable reference substances.

INTRODUCTION

In numerous studies, carrier-free capillary isotachophoresis (ITP) has been found to be a promising analytical tool in peptide chemistry. The basic work of Kopwillem and co-workers[1–4] opened the way for new applications, especially allowing the examination of the purity of chemically synthesized[5–13] and naturally occurring[14–21] peptides.

In the present study, analytical ITP was employed to control carboxypeptidase-Y catalyzed synthesis of a N-protected dipeptide, N-benzoyl-L-alanyl-L-glutamine (N-Bz-Ala-Gln). The peptide N-Bz-Ala-Gln, free amino acids, as well as the side product N-Bz-Ala and contaminating salts could be analyzed in a single experiment. In addition to the qualitative approach, ITP affords information about the composition of the samples without the necessity to concomitantly analyze reference substances, thereby facilitating a simple subsequent quantification. Furthermore, the method allows easy control of the subsequent gel chromatographic purification and, as further benefit, the feasibility directly to evaluate the amino acid composition of the peptide after enzymatic and acid hydrolysis.

EXPERIMENTAL

Materials

All free amino acids and their derivatives were obtained from Sigma (St. Louis,

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MO, U.S.A.). Solvents and reagents (all analytical grade) were provided by E. Merck (Darmstadt, F.R.G.). The enzyme carboxypeptidase-Y (CPD-Y, E.C. 3.4.16.1) was obtained from E. Merck as lyophilized powder (10% enzyme in sodium citrate), exhibiting an activity of 100 U per mg protein. The enzyme was desalted by gel filtration on Sephadex G-25 superfine (25 cm × 1.5 cm, Pharmacia, Uppsala, Sweden) using distilled water as eluent as described by Widmer and Johansen[22]. After lyophilization, an CPD-Y stock solution of 7 mg/ml (100 μM) was prepared and stored at −20°C.

Analytical isotachophoresis

Anionic isotachophoretic analyses were performed by using a 2127 Tachophor (LKB, Bromma, Sweden) with an automatic driving control unit[12]. Separations were carried out in a PTFE capillary (300 mm × 0.55 mm I.D.). Conductivity and UV (254 nm) signals were monitored by employing a two-channel recorder (Kipp & Zonen, Delft, The Netherlands) with a chart speed of 6 cm/min. For detection, the migration current was reduced from 210 to 60 μA. Depending on the chloride concentration of the sample and the chosen terminating electrolyte, the separations required 15–30 min. As leading electrolyte, a 10 mM solution of hydrochloric acid titrated to pH 9.1 with Ammediol was used. The terminating electrolyte buffer contained 10 mM glutamine (electrolyte system I) or β-alanine (electrolyte system II), respectively, adjusted to pH 10 with saturated barium hydroxide solution. These electrolyte solutions were prepared from analytical grade chemicals provided by E. Merck, Sigma and Fluka (Buchs, Switzerland) as described in detail previously[9], and were stored at 4°C.

Enzymatic peptide synthesis

With the aid of a Radiometer (Copenhagen, Denmark) pH-Stat assembly (PHM 84 research pH-meter, TTT/TTA 80 titrator and ABU 80 autoburette), CPD-Y catalyzed peptide synthesis was carried out at pH 9.5 using benzoyl-L-alanine methyl ester (N-Bz-Ala-OMe) as substrate and the free amino acid glutamine (Gln) as nucleophile, as described by Widmer and Johansen[22]. A 0.3 M stock solution of Gln was prepared by dissolving the respective amount in 0.1 M potassium chloride containing 1 mM EDTA. Aliquots (50 μl) of the reaction mixture were taken several times during the reaction period (20 min) and were mixed with 200 μl of 1 M hydrochloric acid to quench the reaction. A 1.5-μl aliquot of each sample was then directly analyzed by isotachophoresis.

The crude peptide material was purified by gel filtration on a glass column (250 cm × 2.5 cm) filled with Sephadex G-10 (Pharmacia) as described earlier[23].

Enzymatic and acid hydrolysis

By adding 0.1 mg aminopeptidase M (E.C. 3.4.11.2, E. Merck), enzymatic hydrolysis of N-Bz-Ala-Gln was performed at pH 7.2 and room temperature in 1 ml sodium phosphate buffer (12.5 mM) containing 1.5 mg of the purified peptide. Isotachophoretic analysis was carried out on an aliquot obtained after 1 h. For acid hydrolysis, 1.0 mg of the synthesized peptide was placed in a glass tube and dissolved in 0.5 ml of 5.7 M hydrochloric acid. Subsequently, the tubes were sealed *in vacuo* and incubated for 24 h at 110°C. The dried hydrolysates were resolved in distilled water and analyzed using β-Ala as terminating electrolyte (electrolyte system II).

RESULTS

Seven specimens of N-Bz-Ala-Gln obtained at intervals during the reaction period were analyzed by applying Gln as the terminating electrolyte. Three of the respective isotachopherograms are compared with an electrolyte run in Fig. 1. Despite barium hydroxide being added to the terminating electrolyte as recommended[24], considerable amounts of hydrogen carbonate formed during the separation were detectable in all analyses performed. The presence of impurities in the electrolyte system is apparent (Fig. 1A). In each sample analyzed at least five zones were detectable, as indicated by the conductivity signal (Fig. 1B–D). Only two zones showed characteristic high UV absorption at 254 nm, suggesting the presence of benzoyl protecting groups.

Four of the solutes present in the reaction mixture could be identified with the aid of reference substances. As illustrated in Fig. 2, the analysis of an equimolar standard mixture of acetate, glutamic acid (Glu), pyroglutamic acid (pGlu) and N-Bz-Ala provided convincing evidence that these substances are identical with those detected in the samples. The remaining UV-absorbing zone by necessity corresponded to the synthesized peptide N-Bz-Ala-Gln, as identified after purification (*vide infra*).

In Fig. 3, an attempt is made to illustrate the time course of the enzyme-catalyzed aminolysis and hydrolysis, respectively. The conductivity zone lengths of both N-Bz-Ala-Gln and N-Bz-Ala rapidly increased during the first 10 min, followed by a steady decrease in the rate of formation of both products. The maximum zone lengths seen after 20 min suggest that the enzymatic reaction is complete. It is also emphasized that the conductivity zone lengths for the three non-UV-absorbing solutes remained essentially unchanged during the reaction period (not depicted in Fig. 3, but indicated in Fig. 1). This finding indicates that the solutes acetate, Glu and pGlu have not participated in the CPD-Y catalyzed reaction.

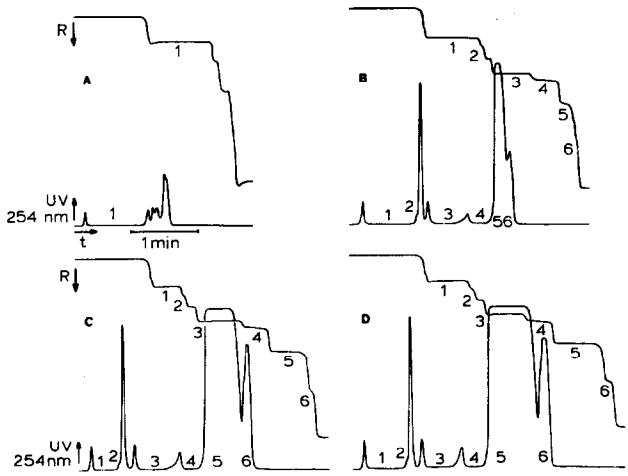

Fig. 1. Isotachophoretic analyses (electrolyte system I) of three different specimens (injection volume 1.5 μl) of N-Bz-Ala-Gln obtained during the reaction period. (A) Electrolyte system; (B) 0.5 min, (C) 5 min and (D) 20 min after addition of CPD-Y. Key: 1 = hydrogen carbonate; 2 = acetate; 3 = Glu; 4 = pGlu; 5 = N-Bz-Ala; 6 = N-Bz-Ala-Gln. R = Increasing resistance.

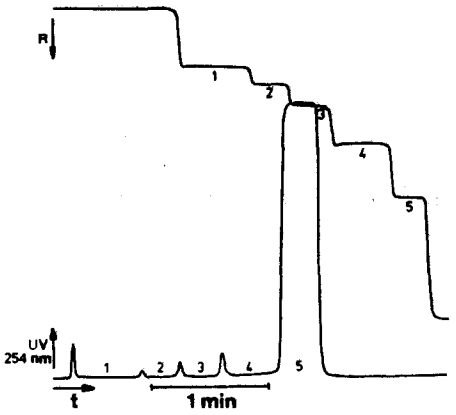

Fig. 2. Isotachophoretic analysis (electrolyte system I) of a reference mixture of acetate, Glu, pGlu and N-Bz-Ala. Injection volume: 10 μl, corresponding to 0.30 μg acetate, 0.74 μg Glu, 0.65 μg pGlu and 0.97 μg N-Bz-Ala. Key as in Fig. 1.

In Fig. 4, isotachopherograms of the crude material (A) and the purified peptide fraction (B) are given. Obviously, a simple gel chromatographic purification step is sufficient to approach 100% enrichment of the peptide N-Bz-Ala-Gln.

Identification of the synthetic product N-Bz-Ala-Gln could be performed after purification by analyzing the acid and enzymatic hydrolysates, respectively. The corresponding isotachopherograms are compared to the isotachophoretic pattern of the electrolyte system in Fig. 5. Although all chemicals used were recrystallized three times, impurities presumable derived from β-Ala were still present in the electrolyte system (Fig. 5A). Analysis of the acid hydrolysate revealed three distinct zones as indicated by the conductivity signal (Fig. 5B), only one of them exhibiting a characteristic UV absorption at 254 nm. As demonstrated in Fig. 5C, enzymatic hydrolysis of N-Bz-Ala-Gln resulted in the formation of one UV-absorbing and one non-UV-absorbing compound, obviously not identical with those determined after acid hydrolysis.

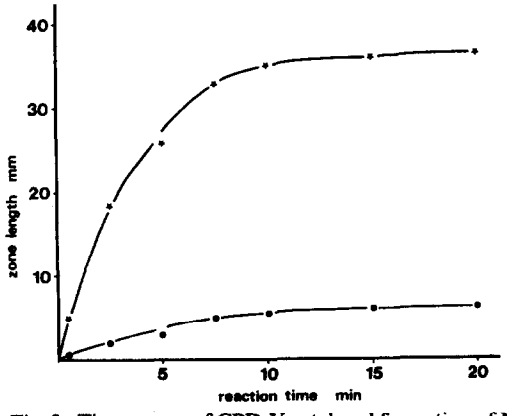

Fig. 3. Time course of CPD-Y catalyzed formation of N-Bz-Ala-Gln (●—●) and N-Bz-Ala (★—★). For synthesis conditions see Experimental.

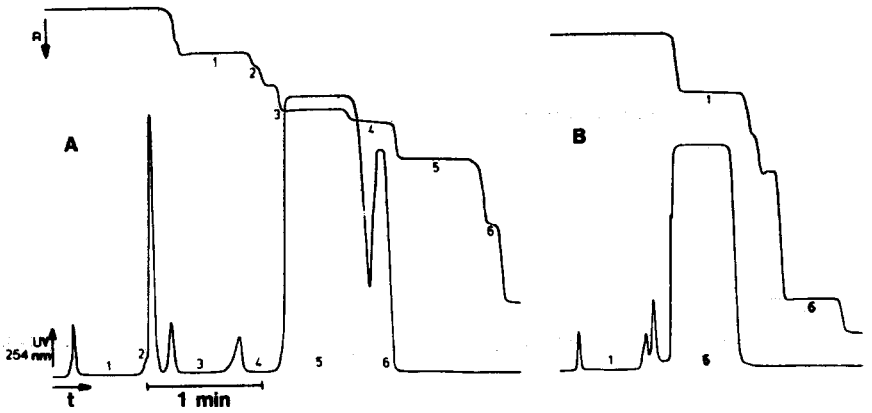

Fig. 4. Isotachophoretic analyses (electrolyte system I) of N-Bz-Ala-Gln before (A) and after (B) gel chromatographic purification. Injection volume: 1.5 μl. Key as in Fig. 1.

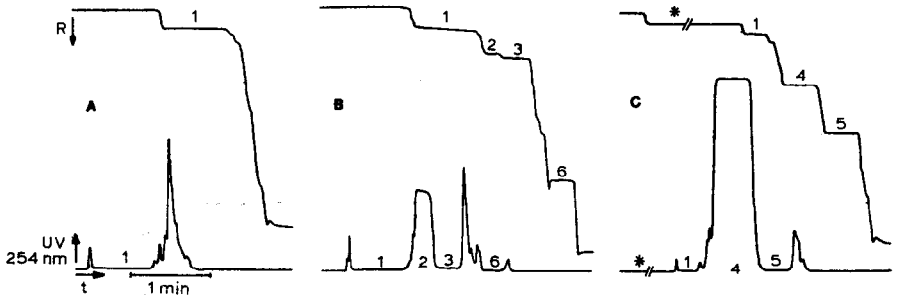

Fig. 5. Isotachophoretic analyses (electrolyte system II) of acid and enzymatic hydrolysates of N-Bz-Ala-Gln. (A) Electrolyte system; (B) acid hydrolysate; (C) enzymatic hydrolysate. Injection volume: 5 μl. Key: 1 = hydrogen carbonate; 2 = benzoate; 3 = Glu; 4 = N-Bz-Ala; 5 = Gln; 6 = Ala; * = phosphate derived from the buffer solution.

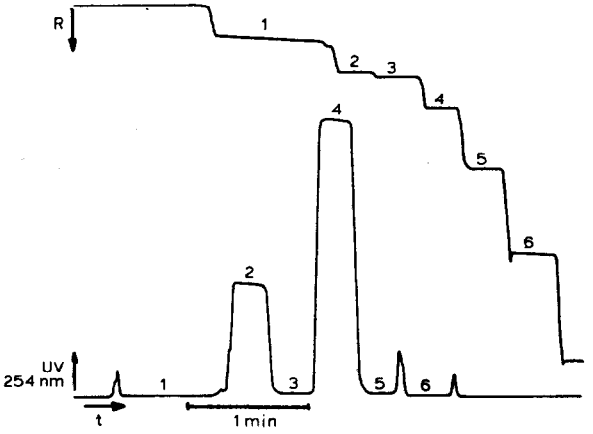

Fig. 6. Isotachophoretic analysis of a reference mixture of acetate, benzoate, Glu, N-Bz-Ala, Gln and Ala. Injection volume: 10 μl, corresponding to 0.30 μg acetate, 0.61 μg benzoate, 0.74 μg Glu, 0.97 μg N-Bz-Ala, 0.73 μg Gln and 0.45 μg Ala. Key as in Fig. 5.

Analysis of an equimolar standard mixture containing benzoate, Glu, N-Bz-Ala, Gln and Ala revealed, as expected, three non-UV-absorbing and two UV-absorbing zones (Fig. 6). These solutes are considered to be possible hydrolysis products of N-Bz-Ala-Gln. A comparison between the characteristic step heights and UV absorptions determined for the reference substances (Fig. 6) and those from the acid and enzymatic hydrolysate (Fig. 5) enabled identification of the respective cleavage products. Both acid and enzymatic hydrolysis fully confirmed the proposed structure of the synthesized peptide.

DISCUSSION

The numerous previous applications of ITP in controlling peptide synthesis suggest the use of this method as tool in controlling the efficacy of enzyme-catalyzed peptide synthesis. Apparently, by this method it is feasible simultaneously to analyze the resulting synthetic peptide as well as free amino acids, amino acid derivatives and contaminating ions present in the reaction mixture. This quality approach, exemplified in Figs. 1–3, allows a direct consideration of the reaction conditions chosen and facilitates the optimization of enzymatic peptide synthesis.

By applying ITP, minor quantities of the synthetic peptide could be directly analyzed in the reaction mixture despite the presence of an extremely high concentration of free Gln. Such an excess might seriously disturb, if not jeopardize, recovery of the peptide when analyzing with alternative electrophoretic[25] or chromatographic[26,27] methods. In the present work, the amino acid Gln itself was chosen as the terminating electrolyte, thus free Gln present in the sample remains in the terminating electrolyte zone and does not disturb the separation of other sample ions migrating in front of the terminating zone. Under these analytical conditions a quantitation of free Gln is not feasible. On the other hand, since only inconsequential amounts of the nucleophile will be consumed during the reaction, one surely may abstain from assessing the concentration of this solute in the reaction mixture.

As outlined under Results, the successful extension of ITP to the determination of the composition of the synthesized peptide N-Bz-Ala-Gln after hydrolysis facilitates its final identification (Figs. 5 and 6). It is emphasized that simultaneous detection of the cleavage products by applying alternative methods based on ion-exchange chromatography and/or reversed-phase chromatography is not possible.

According to the basic principles of ITP[24], there is a linear relationship between the absolute amount of a sample ion injected and the corresponding zone length measured. This means that, under standardized analytical conditions, ITP provides not only qualitative, but quantitative information about the composition of the sample. Generally, an absolute quantitative evaluation requires a calibration for all ionic species present in a sample. On the other hand, a relative quantification of sample ions with similar molecular weights and net charge is appropriate without the necessity to establish calibration curves or calibration constants. This is possible since the conductivity zone length is not influenced by substance-specific properties, *e.g.*, absorption coefficients. Based on these considerations and guided by the time/zone relationship given in Fig. 3, the ratio of the hydrolysis product/peptide formed can be estimated to be 5.8:1.0, which indicates that only about 17% of the substrate N-Bz-Ala-OMe has reacted to give N-Bz-Ala-Gln.

A possible explanation for this low yield may lie in the instability and/or low solubility of Gln. Evidently, a successful proteinase-catalyzed synthesis of peptides requires that the ester substrate used will be subject to aminolysis instead of hydrolysis[28–30]. Since the relative rate of aminolysis to hydrolysis is mainly influenced by the amino acid/water ratio, an high concentration of the amino component (in general 0.5–1 *M*) must be employed[28–30]. Owing to the low solubility of free Gln, a maximum concentration of only 0.3 *M* is applicable. Another factor contributing to the low concentration of Gln in the reaction mixture is certainly the instability of this amino acid, resulting in the formation of pGlu and Glu as indicated in Fig. 1.

It is currently suggested that synthetic short chain peptides are suitable components of future intravenous solutions[31,32]. A prerequisite for a commercial supply of such preparations, however, is the rationale production of short chain peptides on an industrial scale. Since the commonly used chemical syntheses are extremely uneconomic, laborious and inefficient, a biotechnological enzyme-catalyzed production of short chain peptides might be an appreciated alternative method.

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