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Kinetic analysis of biotinylation of specific residues of peptides by high-performance liquid chromatography

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ABSTRACT

A procedure employing C_{18} reversed-phase high-performance liquid chromatography (HPLC) is described for evaluating the kinetics of biotinylation of specific residues of peptides after reaction with N-hydroxysuccinimide esters of biotin. Utilizing this HPLC method, we determined the observed pseudo-first-order reaction rate constant (k'_1) of biotinylation of lysyl residues in two model peptides, [biotinyl-Ser^{108}]ProA-egg laying hormone (108–121) and pGlu–Lys–Trp–Ala–Pro, to be $1.22 \cdot 10^{-2}$ s^{-1} and $1.08 \cdot 10^{-2}$ s^{-1}, respectively, in 0.05 *M* sodium phosphate buffer, pH 8.2, at 25°C. The respective reaction half-lives of the two peptides were 57 s and 64 s. In addition, HPLC analytical methods were established for determining the time-course of hydrolysis of biotinylating reagents at acidic and alkaline pH and for evaluating biotin reagent homogeneity.

INTRODUCTION

Since the earliest days of biochemical research, chemical modification studies have proven to be invaluable for investigating the structure and function relationships of biologically important molecules. One particularly versatile technology, based on the covalent attachment of the vitamin biotin to peptides, proteins, and nucleic acids, has emerged during the last two decades [1]. This technology exploits the strong affinity ($K_a \approx 10^{15}\ M^{-1}$) that is characteristic of the interaction between biotin and the tetrameric egg-white protein avidin. Bioactive compounds which are linked to the valeric acid side chain of biotin typically retain their biological properties. These modified molecules are able to bind to their specific biological targets (*e.g.*, receptors) allowing the biotinyl group to be available for interaction with avidin. Since a variety of reporter groups can be conjugated to avidin, the avidin–biotin system can be utilized in a host of applications involving the localization, visualization, or isolation of specific biomolecules.

An important aspect of the application of the avidin–biotin system is the synthesis of a specific, bioactive, biotinylated protein or peptide. There are a variety of reagents that can biotinylate different functional groups on proteins and peptides. The N-hydroxysuccinimide esters of biotin (NHS-biotin) are a widely used class of biotinylating reagent which are reported to react with primary amino groups to form N-acylated derivatives [1]. Typical-

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ly, these reagents are either simple NHS-biotin esters or related compounds which contain a spacer of 6-aminohexanoic acid between the biotin and NHS moieties. These compounds can also be sulfonated on the NHS moiety to increase their solubility characteristics in aqueous solutions. Although the biotin reagents are widely employed for labeling peptides and proteins they have not been evaluated in depth as chemical modification reagents. Little information is available regarding the kinetics of their reactivity with specific reactive groups of proteins.

In a series of experiments with several different bioactive peptides, we have reported that the NHS-biotin esters can be used to identify differences in the intrinsic reactivity of amino groups [2–5]. Furthermore, we have found that unexpected modifications of seryl and tyrosyl residues can occasionally occur [2]. These experiments have underscored the need to more fully investigate the chemical modification of bioactive peptides and proteins with NHS-biotin esters by conducting a careful analysis of the reaction kinetics of biotinylation with these esters. To this end, we have developed HPLC methods employing a C_{18} reversed-phase column to determine the relative reactivity of NHS-biotin esters during reactions with different model peptides. In this report, we describe these HPLC methods in detail and demonstrate how they may be applied to kinetic analysis of the biotinylation of specific peptide residues. In addition, we have also established HPLC methods to investigate biotinylating reagent homogeneity and the extent of their hydrolysis under described reaction conditions.

EXPERIMENTAL

Materials

Trifluoroacetic acid (HPLC grade), N,N-dimethylformamide, dimethylsulfoxide, sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-εAhx-biotin), succinimidyl-6-(biotinamido) hexanoate (NHS-εAhx-biotin), sulfosuccinimidyl D-biotin (sulfo-NHS-biotin), and succinimidyl D-biotin (NHS-biotin) were obtained from Pierce. NHS-εAhx-biotin was also purchased from Sigma and Molecular Probes. NHS was from Sigma and εAhx-biotin was obtained from Molecular Probes. Acetonitrile (HPLC grade) was obtained from Mallinckrodt. Sodium acetate, sodium phosphate and urea were obtained from Fisher Scientific. Glycine was purchased from Calbiochem. Reagents for amino acid compositional analysis were from Applied Biosystems. Synthetic egg-laying hormone (ELH) [6] and ProA-ELH(108–121) (Ser–Leu–Glu–Ser–Gly–Ile–Ser–Lys–Arg–Ile–Ser–Ile–Asn–Gln–NH_2) [7] were prepared in the Biosynthesis Laboratory of the University of Texas Medical Branch by solid-phase synthesis [8] using a fully automated synthesizer (Biosearch Model 9600). Synthetic peptide, <EKWAP (pGlu–Lys–Trp–Ala–Pro), was obtained from Bachem Bioscience. Vydac C_{18} (5 μm particle size; 300 Å pore diameter) reversed-phase columns (25 cm × 1.0 cm; phase 218TP) and direct connect guard columns containing Vydac C_{18} (5 μm particle size) cartridges were purchased from Alltech Applied Science Labs. An Asahipak ODP-50 C_{18} (5 μm particle size) reversed-phase column (15 cm × 0.6 cm) was purchased from Keystone Scientific, Bellefonte, PA, USA.

Biotinylation reactions

For the preparation of monobiotinyl-αNH_2-ProA-ELH(108–121), sulfo-NHS-εAhx-biotin was weighed and the peptide, dissolved in 0.05 *M* sodium phosphate buffer, pH 8.2, was added to the dry powder. The molar ratio of reagent:peptide was 2.5:1. The biotinylated peptide derivatives were subsequently separated by HPLC on a Vydac semi-preparative C_{18} column (results not shown) and chemically characterized by amino acid compositional analysis and automated peptide sequence analysis. The HPLC fractions containing monobiotinyl-αNH_2-ProA-ELH(108–121) were pooled and lyophilized.

For reagent hydrolysis and kinetic analysis of peptide biotinylation, NHS-εAhx-biotin was dissolved in 25 μl N,N-dimethylformamide just prior to reaction. Tight sealing Whatman 1.5-ml conical screw cap microtubes containing 0.25 mg or 0.5 mg of reagent were prepared by aliquoting reagent (50 μl) dissolved in N,N-dimethylformamide and immediatcly drying by vacuum centrifugation. The biotinylating reagent stored in these tubes was stable for at least 2–3 weeks at 4°C. For kinetic studies, peptides <EKWAP and monobiotinyl-αNH_2-ProA-ELH(108–121) dissolved in 200 μl of 0.05 *M* phosphate buffer, pH 8.2, were added to the biotin

reagent in 25 μl N,N-dimethylformamide and reacted for the times indicated. Reactions were stopped by the addition of 125 μl of 1 *M* glycine–HCl, pH 3.0. The reaction mixtures were injected immediately onto the C_{18} HPLC column. The molar ratio of reagent:peptide was 25:1 for the biotinylation of < EKWAP and 57:1 for the biotinylation of monobiotinyl-αNH_2-ProA-ELH(108–121). For all biotinylation reactions described the NHS-εAhx-biotin used was that obtained from Molecular Probes; however, the NHS-εAhx-biotin obtained from Sigma was also found to be homogenous by HPLC. The stability of NHS-εAhx-biotin in aqueous solution over 90 min was tested at pH 8.2 (sodium phosphate buffer), pH 6.5 (sodium phosphate–trifluoroacetic acid buffer) and pH 4.0 (sodium acetate buffer). The biotinylating reagent (0.25 mg) was dissolved in 25 μl of N,N-dimethylformamide and 200 μl of buffer was added. At designated times 0.5 ml of 0.1% trifluoroacetic acid was added and the mixture was immediately injected onto the HPLC column. All above reactions were conducted at 25°C.

High-performance liquid chromatography

A Beckman Model 332 gradient liquid chromatograph with an on-line Hitachi Model 100-40 variable-wavelength spectrophotometer was used for most of the studies. A second Model 332, which was on-line with a Beckman Model 160 fixed-wavelength absorbance detector, was also employed.

Reaction mixtures containing biotinylating reagents or biotinylated peptides were applied to a Vydac C_{18} reversed-phase HPLC column and eluted with a linear gradient of solvent A (0.1% trifluoroacetic acid) and solvent B (100% acetonitrile containing 0.1% trifluoroacetic acid) at a flow-rate of 1.75 ml/min. The column eluate was monitored at 215 nm and 1.0-min fractions were collected. Fractions were pooled based on absorbance and were subjected to amino acid compositional analysis. All fractions were dried by vacuum centrifugation and stored at 4°C.

The linear gradient employed to fractionate biotinylating reagents and biotinylated peptides is shown in the top figure panel for each experiment. Three gradient programs were used for these studies. The gradient program for evaluating reagent homogeneity and hydrolysis was simply 5% acetonitrile to 50% acetonitrile over 60 min. To fractionate the biotinylation reaction products of [biotinyl-Ser^{108}]ProA-ELH(108–121) the HPLC program was 16% isocratic acetonitrile for 10 min whereupon the gradient was increased from 16% acetonitrile to 50% over 110 min. To separate the biotinylation reaction mixtures of the < EKWAP peptide the gradient conditions were 10% acetonitrile to 55% over 60 min.

Amino acid analysis

The amount of biotin was determined by amino acid analysis of 6-aminohexanoic acid as we previously described [3]. Samples of those NHS-biotin esters containing a 6-aminohexanoic acid spacer arm were applied directly to an Applied Biosystems 420H (derivatizer/hydrolyzer) amino acid analyzer with on-line acid hydrolysis and pre-column phenylthiocarbamyl derivatization.

RESULTS AND DISCUSSION

We have previously reported that biotinylated peptide derivatives were readily separated by C_{18} reversed-phase HPLC [2,4]. In fact, we have found that the resolution of various biotinylated peptide derivatives was consistently remarkable for a number of peptides investigated. For example, the monobiotinylated derivatives of the three amino groups (αNH_2-Ile^1; εNH_2-Lys^8 and εNH_2-Lys^{36}) of ELH of *Aplysia* can be well separated on a Vydac 300 Å C_{18} column (Fig. 1A) [4]. To inquire whether this resolution was unique to the Vydac column, a comparative elution profile of the biotinylation reaction products of ELH was made using an Asahipak ODP-50 column packed with a C_{18} modified polymer gel (Fig. 1B). The elution profile from the Asahipak column was essentially comparable to that of the Vydac column except that the elution of fractions 4 and 5 from the Asahipak column was reversed (Fig. 1A and B). The observed high degree of resolution of biotinylated peptides was therefore not likely due to interactions with the base material support since the Vydac column contained a silica gel base and the Asahipak column contained a polymer base support. We have consistently observed that the elution pattern of peptides after biotinylation typically demonstrated increased bandwidths, similar to that observed during isocratic elution conditions, suggesting a relatively strong in-

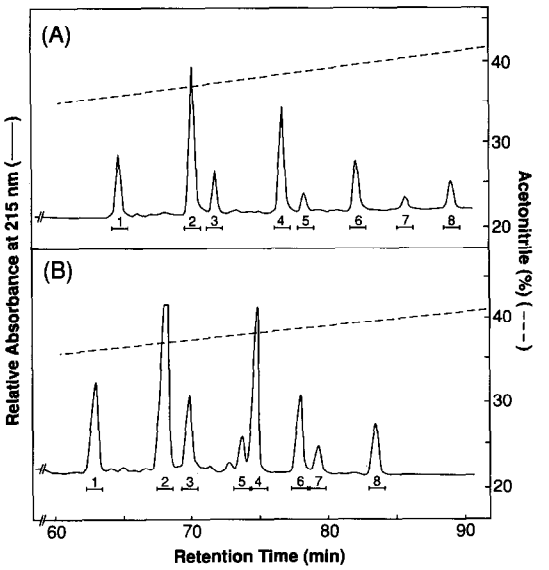

Fig. 1. Comparative C_{18} reversed-phase HPLC separation of a reaction mixture of NHS-εAhx-biotin with *Aplysia* egg-laying hormone. Biotinylating reagent was dissolved in 25 μl N,N-dimethylformamide and 225 μl of 0.05 *M* $NaHCO_3$, pH 8.2, containing 15 nmol (66 μg) of ELH, was added. The molar ratio of reagent to peptide was 8:1. After 30 min of reaction at 25°C the pH was lowered to 6.0 by addition of 1 ml of 20% acetonitrile in 0.1% trifluoroacetic acid and the reaction mixture was immediately injected onto the HPLC column. (A) Vydac semipreparative column, flow-rate 1.75 ml/min); (B) Asahipak ODP-50 column, flow-rate 1.0 ml/min. Gradient conditions (identical for both columns) and the chemical characterization of each fraction were as we previously reported [4].

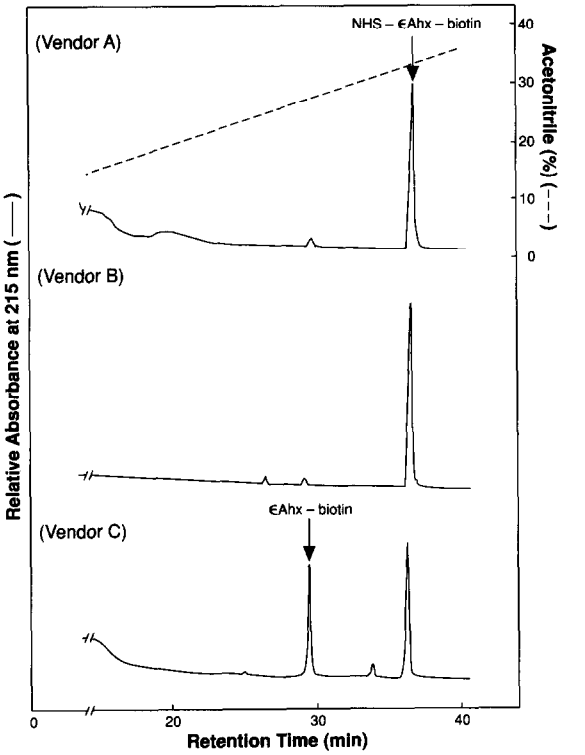

Fig. 2. C_{18} reversed-phase HPLC isolation of NHS-εAhx-biotin obtained from three vendors on a Vydac semipreparative column. Flow-rate was 1.75 ml/min. Biotinylating reagent (0.25 mg) was dissolved in 25 μl N,N-dimethylformamide, followed by 0.5 ml 0.1% trifluoroacetic acid and injected immediately onto the HPLC column. Reagent obtained from vendor C was considerably hydrolyzed as evidenced by the εAhx-biotin peak.

teraction between the biotinylated solute and the C_{18} bonded phase. The covalent addition of a single biotin group usually significantly increased the retention time of the peptide [2,4]. Furthermore, even with proteins, we have observed that small differences in the number of biotinyl groups can be reflected in significant retention time differences [3].

Prior to conducting kinetic studies of biotinylation of peptides we evaluated the homogeneity of a number of available NHS-biotin esters including the sulfonated derivatives. These initial studies were important since we had previously observed some variation of peptide biotinylation that we had attributed to the quality of the biotinylating reagents [2]. Evaluation of biotinylating reagent homogeneity is shown in Figs. 2 and 3. Fig. 2 compares the C_{18} HPLC elution profile of NHS-εAhx-biotin obtained from three vendors. All reagents were analyzed within 2 weeks of their receipt from vendors and were stored dessicated at 4°C. As indicated in Fig. 2, the product obtained from vendor C contained substantial amounts of εAhx-biotin, presumably as a result of hydrolysis during manufacture or subsequent storage. Various lots of reagents purchased from vendor C at different times, no more than one year prior to these studies, varied 40–60% in the amount of εAhx-biotin present. The quantitation of NHS-εAhx-biotin and εAhx-biotin was achieved by amino acid analysis of the 6-aminohexanoic acid spacer arm as we previously described [3]. The observed A_{215nm} molar absorptivity was higher for NHS-εAhx-biotin than εAhx-biotin by a factor of 1.34:1.

In a separate series of analyses, using HPLC con-

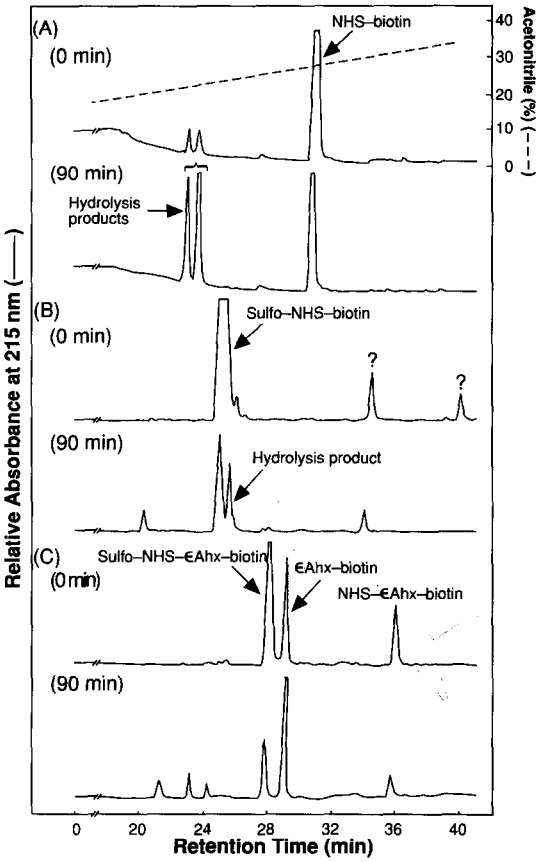

Fig. 3. Evaluation of other biotinylating reagents and 90-min hydrolysis products by C_{18} reversed-phase HPLC on a Vydac semipreparative column. Flow-rate was 1.75 ml/min. Non-sulfonated biotinylating reagents (0.5 mg) were dissolved in 25 μl N,N-dimethylformamide followed by addition of 200 μl 0.05 *M* sodium phosphate buffer, pH 8.2. For sulfonated reagents, phosphate buffer was added to 0.5 mg dry reagent. For 0 time of reaction, 0.5 ml of 0.1% trifuloroacetic acid was added prior to the buffer; for the 90-min reaction time the acid was added at 90 min.

ditions identical to those described for the separation of NHS-εAhx-biotin, we also evaluated the homogeneity and hydrolysis products of other NHS-biotin esters (Fig. 3). Fig. 3A shows the elution of NHS-biotin at time 0 and after 90 min of hydrolysis. The two hydrolysis products indicated by arrows were not identified. Fig. 3B illustrates a similar analysis of sulfo-NHS-biotin. The only obvious hydrolysis product evident in this reaction was a peak which eluted immediately after the reagent. The absorbance of this hydrolysis product, however, was considerably lower than expected when compared to comparable products from other esters.

Analysis of sulfo-NHS-εAhx-biotin (Fig. 3C) indicated that this reagent contained εAhx-biotin and suprisingly some NHS-εAhx-biotin. In these analyses special attention was paid to preventing carryover and reagent contamination. A blank run conducted immediately prior to analysis indicated no evidence of NHS-εAhx-biotin. Also, the injection port was well rinsed before analysis. Confirmation that the contaminant peak was in fact NHS-εAhx-biotin was supported by three observations: (1) the retention time was identical to authentic NHS-εAhx-biotin; (2) amino acid analysis confirmed the presence of 6-aminohexanoic acid; and (3) the peak diminished after 90 min of hydrolysis at pH 8.2. Taken together these results established the utility of C_{18} reversed-phase HPLC for evaluating the homogeneity of commercial or otherwise obtained NHS-biotin esters. Clearly, the quality of some of the commercially available NHS-biotin esters was variable. Furthermore, in view of the fact that these esters can hydrolyze during storage, this HPLC procedure also offers a convenient method of measuring the extent of spontaneous reagent hydrolysis. It is also of value to point out that HPLC analysis of authentic NHS indicated that it eluted with the salt front early in the gradient elution program.

In order to establish the kinetics of biotinylation of our model peptides it was necessary to ensure that our reaction conditions were pseudo-first-order and that we were sufficiently in excess in regard to concentration of the selected biotinylating reagent, NHS-εAhx-biotin. Since the hydrolysis of NHS-εAhx-biotin was an important factor, we investigated the extent of hydrolysis of the reagent under our described reaction conditions using HPLC. Fig. 4 shows the time-course of hydrolysis of NHS-εAhx-biotin at pH 8.2, 6.5 and 4.0. As indicated, the rate of hydrolysis was significantly slower at acid pH. Importantly, these results indicated that over the time-course of our peptide kinetic studies the extent of reagent hydrolysis was not sufficiently large to influence our reaction kinetics and that we could readily maintain reagent excess. Fig. 5A shows three representative chromatograms of HPLC analysis of selected reaction times of spontaneous reagent hydrolysis at pH 8.2.

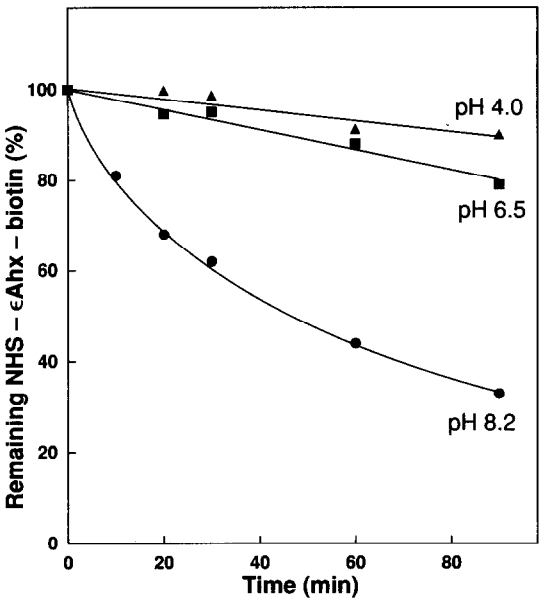

Fig. 4. Time-course of hydrolysis at 25°C of NHS-εAhx-biotin at pH 4.0 (▲), 6.5 (■), and 8.2 (●). Conditions were as described under Experimental.

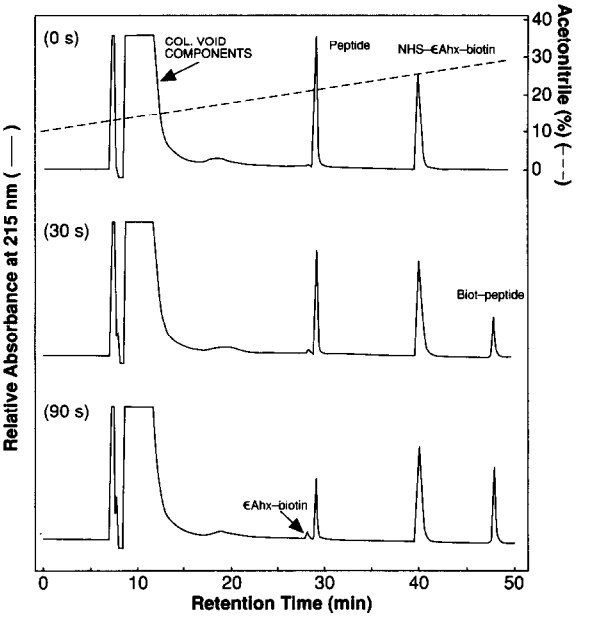

Fig. 6. Representative chromatograms from Vydac C_{18} reversed-phase HPLC analysis of the time-course of biotinylation of 21.8 nmol (13.3 μg) of the peptide < EKWAP by NHS-εAhx-biotin at 25°C for times shown. "Biotin-peptide" (30 s and 90 s panels) was monobiotinylated on Lys^2.

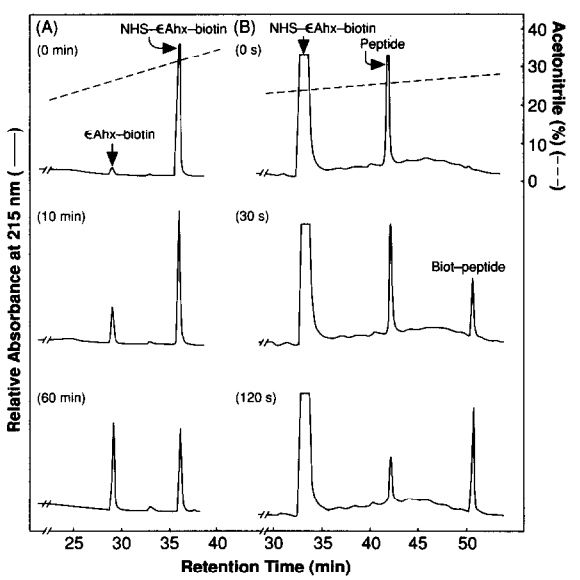

Fig. 5. Representative chromatograms from Vydac C_{18} reversed-phase HPLC analysis of (A) the time-course of hydrolysis of 550 nmol (0.25 mg) of NHS-εAhx-biotin at reaction times indicated and (B) the time-course of biotinylation of 9.7 nmol (18.1 μg) of monobiotinyl-αNH_2-A-ELH(108–121) by NHS-εAhx-biotin at 25°C for times indicated.

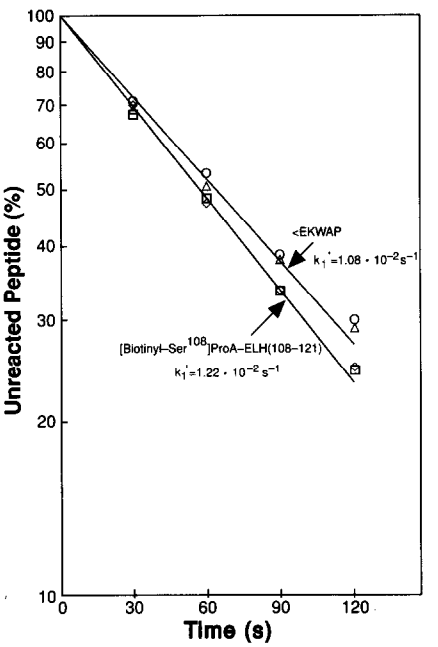

Fig. 7. Observed pseudo-first-order reaction kinetics of the two peptides shown with NHS-εAhx-biotin at 25°C. Duplicate reaction times are indicated for each curve: □, ◇ = [biotinyl-Ser^{108}]ProA-ELH(108–121); ○, △ = < EKWAP.

The kinetics of biotinylation of two different lysyl residues in two model peptides are described in Figs. 5B, 6 and 7. HPLC chromatograms of representative time-course biotinylation reactions of [biotinyl-Ser^{108}]ProA-ELH(108–121) are presented in Fig. 5B. Similarly, representative chromatograms of biotinylation of the peptide pGlu–Lys–Trp–Ala–Pro are given in Fig. 6. For a representative comparison the results given in Fig. 6 show the entire chromatograms, whereas, for convenience, the early elution regions of all previous chromatograms were abbreviated. Fig. 7 gives the observed pseudo-first-order log plots of biotinylation of the lysyl εNH_2 groups of the two model peptides shown. The observed k'_1 values for [biotinyl-Ser^{108}]ProA-ELH (108–121) and <EKWAP were $1.22 \cdot 10^{-2}$ s^{-1} and $1.08 \cdot 10^{-2}$ s^{-1}, respectively. The k'_1 values obtained for the two peptides were similar and likely represented the reactivity of a fully exposed εNH_2-group since the reactivity of <EKWAP was identical in 8 *M* urea (results not shown). The reaction half-life for [biotinyl-Ser^{108}]ProA-ELH(108–121) was 57 s and for <EKWAP was 64 s. The observed reactivities of the εNH_2-groups of these two peptides was somewhat faster than that observed for the reaction of 2,4,6-trinitrobenzenesulfonate with the εNH_2-groups of glycyllysine in 0.05 *M* sodium borate buffer, pH 9.5. The half-life for the two peptide reactions with NHS-εAhx-biotin averaged to be *ca.* 61 s (pH 8.2) whereas that reported for the reaction of glycyllysine with 2,4,6-trinitrobenzenesulfonate was 105 s (pH 9.5) [9].

The above described HPLC method for determining reaction rates of amino acid residues of peptides with the NHS-biotin esters clearly offers a number of advantages. These include (1) evaluation of biotinylating reagent quality, (2) determination of the extent of spontaneous reagent hydrolysis, (3) simultaneous measurement of the amounts of all reaction components including reagent, reagent hydrolysis products, unmodified peptide and biotinylated peptide, (4) reasonably high sensitivity using low nmol amounts of peptide and (5) potential for comparative kinetic analyses. An additional advantage of using the NHS-εAhx-biotin reagent is that it allows for accurate measurement of all reaction components by quantitation of the amount of 6-aminohexanoic acid spacer arm by amino acid analysis [3]. Peptides that contain mixtures of reactive groups can be initially reacted under more limiting reagent conditions to obtain suitable biotinylated peptides with only a single reactive group [2,4].

In summary, we have established HPLC methods for characterizing the kinetics of biotinylation of peptides using NHS-biotin esters. Also included in these studies are chromatographic procedures for evaluating reagent homogeneity and determining the extent of biotinylating reagent hydrolysis.

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