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High-Sensitivity Isoelectric Focusing of Biotinylated Peptides: A New Method[1]

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A novel technique to selectively analyze prelabeled peptides by isoelectric focusing (IEF) is presented. The conditions are described for biotinylation of peptides, their separation in polyacrylamide gels by IEF, and their fixation to the gel matrix with glutaraldehyde. The gels are developed by a color reaction catalyzed by an avidin-coupled enzyme. The technique is suitable for peptides with at least one free amino group or guanidino group after N-terminal biotinylation. The presence of other peptides or proteins does not interfere with the detection. The sensitivity is below 10 pmol, representing a 1000-fold improvement over existing techniques for analyzing low molecular weight peptides by IEF. © 1988 Academic Press, Inc.

KEY WORDS: isoelectric focusing; peptide; biotinylation; polyacrylamide gel; avidin–enzyme staining; angiotensin II.

Isoelectric focusing in polyacrylamide gels represents a powerful analytical and preparative tool in protein chemistry. The adaptation of this technique to peptides, however, met with a number of technical difficulties. While proteins can be fixed to the polyacrylamide matrix by trichloroacetic acid, peptides with a molecular weight below 3000 will be washed out under these conditions (1). Fixation with formaldehyde or glutaraldehyde prior to staining crosslinked both the peptides and the carrier ampholytes (2) because of their polyamino–polycarboxylic acids. Different stains have been used to detect peptides in IEF gels. Conventional protein stains such as Coomassie brilliant blue failed with small molecular weight peptides because they could not be precipitated with trichloroacetic acid (3). The staining technique of Blakesly and Boezi (4) for disc gel electrophoresis detected only peptides above 15 amino acids with a relatively low sensitivity (20 μg). The use of other reagents for staining proteins or amino groups such as permanganate, Lowry reagent, or ninhydrin was limited because of a high background from reacting carrier ampholytes (e.g., (3)). The latter did not interfere with iodine vapors but the detection sensitivity for peptides was low (20–25 μg) (5). Spray reagents specific for arginine, tyrosine, histidine, tryptophan, methionine, and cysteine were limited to peptides containing these amino acid residues (6) and the detection limit was also in the range of 10–20 μg of peptide. Some of the above limitations could be resolved by the technique of Trah and Schleyer. Coomassie brillant blue G-250 dissolved in a formaldehyde–methanol water mix stained a wide range of peptides without the interference of carrier ampholytes. This technique was applied to proteins and peptides down to a molecular weight of 1600 at a sensitivity of 20 μg (7).

In general, the application of the above techniques was limited to peptides above

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1500 Da and a sensitivity of about 20 μg. In immobilized pH gradients the sensitivity was improved by 10-fold and dipeptides became detectable (3).

For direct visualization in the uv after IEF,[3] Kopwillem *et al.* (8) prelabeled synthetic peptides with dinitrophenylhistidine. We report here a novel technique to specifically detect prelabeled low molecular weight peptides in a mixture of peptides and proteins with a sensitivity 1000-fold higher than that of other existing IEF techniques. The conditions are described for biotinylation of peptides, their fixation with glutaraldehyde after IEF, and their *in situ* visualization with a color reaction catalyzed by an avidin-coupled enzyme.

MATERIALS AND METHODS

Materials

Angiotensin II (ATII; Asp–Arg–Val–Tyr–Ile–His–Pro–Phe; M_r 1169), angiotensin I (ATI; Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu; M_r 1406), EGF(aa20–31) (Cys–Met–His–Ile–Glu–Ser–Leu–Asp–Ser–Tyr–Thr; M_r 1297), and the tetrapeptides Trp–Gly–Gly–Tyr (WGGY; M_r 482) and Gly–Gly–Tyr–Arg (papain inhibitor peptide, GGYR; M_r 451) were purchased from Bachem (Bubendorf, Switzerland), and the dipeptide Gly–Gly (GG; M_r 132) was from E. Merck (D-6100 Darmstadt, FRG). The Enzotin biotinylating reagent was obtained from Ortho Diagnostic Systems (D-6903 Neckargemünd, FRG). Glutardialdehyde, dimethylformamide (DMF), Triton X-100, bovine serum albumin (pure, BSA), and the materials and chemicals used for IEF (bisacrylamide premix, 3% ammonium persulfate plot concentrate, gel preparation kit, gel-fix) were supplied by Serva (D-6900 Heidelberg, FRG). Avidin-conjugated alkaline phosphatase (AP), avidin-coupled horseradish peroxidase (POD), aminoethyl carbazol (AEC), 5-bromo-4-chloroindolylphosphate (BCIP), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT) were obtained from Sigma (D-8028 Taufkirchen, FRG) and chymotrypsin was from Boehringer (D-6800 Mannheim, FRG). Casein, ethylenediaminetetraacetic acid, hydrogen peroxide, and the buffer salts were analytical grade from E. Merck.

AEC, MTT, PMS, and BCIP are carcinogens or suspected carcinogens and should be handled accordingly.

Buffers and Solutions

(1) Detection with horseradish peroxidase. The predetection buffer was an aqueous solution of 0.1 M sodium acetate, pH 4.5. The washing buffer (pH 4.5) included in addition 0.5% Triton X-100, 1 mM EDTA, and 0.1% BSA. The staining solution was prepared with 100 ml predetection buffer substituted with 25 μl 30% hydrogenperoxide and 40 mg AEC dissolved in 2 ml DMF.

(2) Detection with alkaline phosphatase. The predetection buffer (pH 8.8) contained 0.1 M Tris/HCl, 0.1 M sodium chloride, and 5 mM magnesium chloride. The washing buffer included in addition 0.5% Triton X-100, 1 mM EDTA, and 2% casein. The staining solution was prepared from 100 ml staining buffer to which 75 μl MTT solution and 150 μl 5% (w/v) BCIP in DMF were added. The MTT solution was prepared by suspending 75 mg MTT in 0.7 ml DMSO and adding 0.3 ml distilled water to complete solubilization.

[3] Abbreviations used: aa, amino acid sequence; AEC, aminoethyl carbazole; AP, alkaline phosphatase; ATI, angiotensin I; ATII, angiotensin II; BCIP, 5-bromo-4-chloroindolylphosphate; BSA, bovine serum albumin; DDW, double-distilled water; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; GG, Gly–Gly; GGYR, papain inhibitor peptide (PIP); IEF, isoelectric focusing; MTT, thiazolyl blue; POD, horseradish peroxidase; WGGY, Trp–Gly–Gly–Tyr; ELISA, enzyme-linked immunosorbent assay; PMS, phenazine methosulfate.

Methods

Biotinylation procedure. Five milligrams of peptide was dissolved in 1 ml of 0.1 M NaHCO3, pH 7.5. In the case of peptides without arginyl or lysyl residues the pH was adjusted to 8.5. The peptide solution was incubated for 48 h at 4°C with a 1.5-fold molar excess of the biotinylating reagent Enzotin (1–12 mg) dissolved in 120 ml of DMSO. The DMSO was removed by lyophilization.

Enzotin is the *N*-hydroxysuccinimide ester of *o*-biotinyl-ϵ-aminocaproic acid (M_r 454). This activated ester reacts with amino groups of amino acids, peptides, or proteins to form a peptide bond (9). The ϵ-aminocaproic acid serves as a spacer to facilitate avidin binding (10).

Isoelectric focusing. Precast polyacrylamide gels (0.15 mm thick) were used as well as self-prepared gels of the same thickness containing 5% acrylamide, 3% cis(*N,N*-methylenebisacrylamide), and 3% Servalyte carrier ampholytes, pH 3–11. Samples of 10 μl or less were applied with an application strip (Serva). Cathode and anode strips were soaked with the cathodic fluid (1.09 g arginine anhydrous base, 0.91 g anhydrous lysine, and 30 ml ethylenediamine in 250 ml DDW) and anodic fluid (0.83 g aspartic acid and 0.92 g glutamic acid in 250 ml DDW), respectively. IEF was performed on a LKB Multiphor II system (LKB, Bromma, Sweden) with a LKB Macrodrive 5 power supply.

Prior to IEF, excess proteins were heat precipitated. The samples were boiled for 3 min, ice-cooled, and centrifuged for 10 min at 8000*g*.

Fixation. After IEF, the peptides were fixed for 30 min in an aqueous solution of unbuffered 10% glutaraldehyde. For peptides which must be crosslinked to the gel matrix via guanido groups of arginyl residues, the reaction requires alkaline conditions such as 0.05 M sodium bicarbonate buffer, pH 9. The gels were washed for 15 min in three changes of 100 ml washing buffer to remove excess glutardialdehyde and then washed twice for 10 min in 500 ml distilled water. The gels were then swollen overnight in 500 ml distilled water at 4°C.

Staining methods. The preswollen gels were incubated for 2 h at 4°C with 100 ml of staining buffer containing 20 U of avidin-conjugated enzyme. Excess enzyme was then removed by washing five times for 5 min with 100 ml of washing buffer and twice for 5 min in 100 ml of predetection buffer. After this extensive washing procedure the gels were developed in the staining solution for 30 min (POD) or 3 h (AP). The POD-stained peptides appeared as narrow brown–red bands, whereas the peptides stained by AP gave lilac bands. Finally, the gels were washed for 5 min in 0.05% Triton X-100 and rinsed with water. The gels were air-dried at room temperature and scanned at 520 nm using a Beckmann CDS-200 computing densitometer system.

HPLC. The HPLC analyses were performed on a Waters 721 WISP. The probes were applied on a Nucleosil C18 4.6 × 250-mm column (particle size 5 μm) using a Waters 600 multisolvent delivery system. The samples were eluted for 30 min with a 10–60% acetonitrile gradient (flow rate 1 ml/min) and detected with a Waters Lambda-Max LC spectrophotometer at a wavelength of 220 nm. The data were stored and processed by a Waters 740 data module.

RESULTS

Conditions for Biotinylation

For optimal reactivity with proteins a 3-fold molar excess of Enzotin at pH 8.5 incubated at 4°C overnight is recommended (Ortho Diagnostic, technical sheet 401). Under these conditions human angiotensin II was biotinylated at the N-terminal and at the guanidino group of arginine as shown by HPLC. This product became insoluble in aqueous solutions. In the presence of a 1.5-fold molar excess reagent and at pH 7.5 the guanidino group of arginine was largely pro-

tected from biotinylation and hence solubility remained unaffected. After 48 h at 4°C the yield was 80% including 6% dibiotinylated peptide as determined by HPLC. Thus, by lowering the pH from 8.5 to 7.5 and by reducing the molar excess from 3.5 to 1.5, the biotinylation reagent undergoes a reaction with a limited selectivity toward amino groups as opposed to guanidino groups (of arginin). These modified conditions were also suitable for the biotinylation of other peptides such as GG, WGGY, GGYR, EGF(aa20–31), ATI, and ATII with molecular weights ranging from 132 to 1296. After biotinylation DMSO can be removed by evaporation in a lyophilizer.

Conditions for Fixation

Biotinylated peptide was applied at 0.1 mg per lane. After IEF the peptides were fixed with 0.5, 2.5, 5.0, 10.0, and 25% unbuffered glutardialdehyde for 30 min at room temperature. Fixation with 10% glutardialdehyde was optimal (data not shown). With lower concentrations the fixation was incomplete; higher concentrations resulted in a higher background. For peptides which after N-terminal biotinylation contain only guanidino groups susceptible to fixation, 10% glutaraldehyde buffered with 0.05 M bicarbonate was used. The optimal pH range was found to be above 8.

Conditions for Staining

The washing buffer, the predetection buffer, and the staining solutions were based on the recommendations provided with the DETEK system (technical sheet Bio-note 314 (AP) and 306 (POD) Ortho Diagnostic Systems), a detection system for biotinylated proteins after immunoblotting based on avidin-conjugated alkaline phosphatase or peroxidase. We have adapted the method for staining peptides. Among the BSA concentrations tested (0.1, 0.5, 2.0, 10.0%), the washing buffer with 0.1% BSA gave the best result for staining with POD. Before AP staining, 2% BSA in the washing buffer was optimal. In the case of AP but not POD, BSA can be replaced by inexpensive casein. Substituting BSA or EDTA/Triton X-100 with 3% gelatin and 0.05% Tween in the washing buffer gave a similar background. Several concentrations (0.1, 0.2, 0.5, 1, 2, 5 U/ml) of the enzymes were tested. The concentrations indicated under Materials and Methods gave the best detection sensitivity for the lowest background. With higher concentrations nonspecific adsorption, presumably to fixed carrier ampholytes and/or the gel matrix, resulted in a wavy background. Excessive washing with higher concentrations of Triton X-100 leads to a weak staining intensity.

In a similar experiment avidin-conjugated β-galactosidase did not catalyze a color reaction, presumably because this high molecular weight conjugate does not sufficiently penetrate the gel. When the gels are dried immediately after staining, a uniform background stain appears. This is due presumably to the light sensitivity of AEC or MTT in the staining solutions. Several washing steps with Triton X-100 and DDW (see Materials and Methods) after staining are sufficient to eliminate this background.

Under the conditions determined here and described under Materials and Methods, 0.01 μg of biotinylated angiotensin II was detectable after electrofocusing and staining with AP (Fig. 1). The sensitivity of the POD technique was 0.05 μg peptide (Fig. 2). After scanning, the peak areas of the peptide bands are proportional to the quantity of peptide applied, after both AP and POD staining (Fig. 3). The advantage of the latter staining procedure was its speed (30 min compared to 1–4 h for AP) and its lower background. By comparison with a protein standard mixture (Protein Test Mix 9, Serva) the isoelectric point of of the biotinylated ATII was 7.8. The unconjugated peptide, stained with Coomassie blue according to Trah and Schleyer (7), displayed a p*I* of 4.8. In the experiments shown in Figs. 1 and 2 excess biotinylation reagent (454.4 Da) was re-

moved by exclusion chromatography on a Sephadex G-10 column. The gels shown have an effective length of 12 cm. By using "minigels" (5 × 5 cm) we were able to reduce the time required for electrofocusing from 90 to 31 min (Serva data sheet "Miniature IEF on Servalyte precotes").

Applications

The above conditions were also suitable for analyzing other biotin-conjugated peptides such as GG, WGGY, GGYR, ATI, and EGF(aa20–31), with molecular weights ranging from 132 to 1296 (data not shown). In the case of GG and WGGY a negative staining was observed. The latter two peptides do not contain residues susceptible to fixation, allowing the diffusion of the peptides and resulting in elevated background staining. We did not, however, investigate why the primary location of the peptide band did not stain and emerged as a negative band.

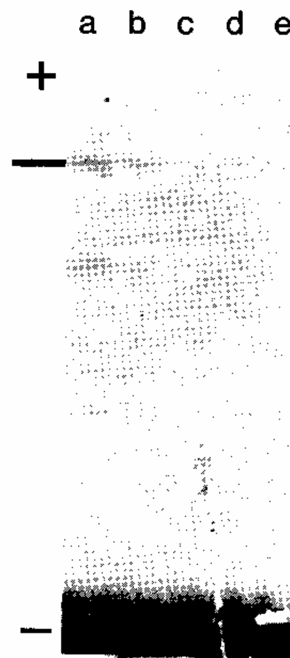

FIG. 2. Staining of an IEF polyacrylamide gel (Servalyte pH 4–9T) with AP. (a) 1, (b) 0.5, (c) 0.1, (d) 0.05, and (e) 0.01 μg biotinylated angiotensin II (—). The anode and cathode are indicated by + and –, respectively.

FIG. 1. Staining of an IEF polyacrylamide gel (Servalyte pH 4–9T) with POD. (a) 1, (b) 0.5, (c) 0.1, (d) 0.05, and (e) 0.01 μg biotinylated angiotensin II. The anode and cathode are indicated by + and –, respectively.

In the following experiment 0.375 mg biotinylated ATII dissolved in 150 μl 100 mM sodium hydrogen phosphate buffer (pH 7.0) was digested with 9 U chymotrypsin (0.1 mg). After 1, 5, 12, 15, 20, and 30 min 25-μl aliquots were removed and heat precipitated. Ten microliters was analyzed by IEF, stained by POD–avidin, and scanned as described. The peak areas plotted against the reaction time enabled monitoring of the enzymatic reaction with the biotinylated peptide as substrate (Fig. 4).

The polyacrylamide gel of Fig. 5 shows that a peptide mixture containing ATII, GGYR, WGGY, and GG (6.25 μg each) did not interfere with the separation and staining of the biotinylated peptide ATII (lane 2). The presence of 25 μg BSA contributed to a slight smearing, presumably due to adsorption of biotinylation reagent to BSA (lane 3). Heat

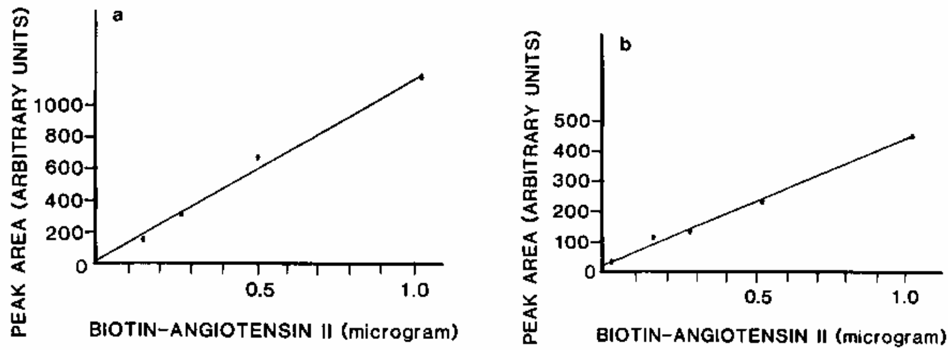

FIG. 3. Calibration curves of AP- (a) and POD (b)-stained biotinylated ATII peptide bands. The peak areas are plotted against the quantity of applied peptide.

precipitation of BSA restores the original sharpness of the peptide band (lane 4). Samples containing 25 μl of heat-precipited serum (2 mg protein; lane 5) or 1.0 mg chymotrypsin (Fig. 4) gave well-focused peptide bands. In the gel of Fig. 5, the heavy band toward the cathode represents the excess biotinylation reagent; the mono- and dibiotinylated ATII migrated toward the anode. The bands were identified by HPLC.

DISCUSSION

In our studies with synthetic peptides as substrates for enzymatic reactions the need for a simple and sensitive technique for the separation and detection of these peptides and their products emerged. Typically the reaction takes place in the presence of mixtures of proteins and peptides such as serum or cell lysates; it is therefore required that irrelevant proteins or peptides do not interfere with the

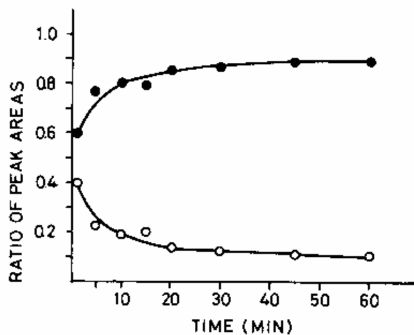

FIG. 4. Degradation of biotinylated ATII by chymotrypsin. Aliquots were removed from the reaction mixture at different time intervals, analyzed by IEF, and scanned. The peak areas of the extinction (ΔE at 520 nm) of the undegraded (○) and degraded (●) peptide are expressed as percentages of the total (for each aliquot) and plotted against reaction time.

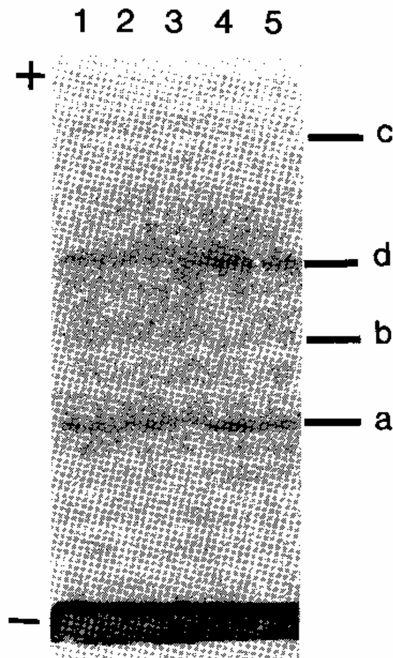

FIG. 5. Isoelectric focusing on Servalyte precotes pH 3–10 of biotinylated ATII (25 μl) only (lane 1) or in the presence of a peptide mixture (ATII, GGYR, WGGY, GG, 6.25 μg each; lane 2), 25 μg BSA (lanes 3 and 4), or 25 μl human serum (lane 5) before (lane 3) and after (lanes 4 and 5) heat precipitation. The anode and cathode are indicated by + and −, respectively. (a) Excess biotinylation reagent, (b) application site of probes, (c) dibiotinylated ATII, (d) monobiotinylated ATII.

detection. The available techniques for the analysis of peptides by IEF detect at best 10 nM peptide and are mostly limited to peptides with a molecular weight above 1500. They all lack the sensitivity required for the analysis of peptides in the picomole range.

In order to reach this level of sensitivity we have made use of the high affinity of avidin to biotin. This system has been used in protein chemistry to isolate biotinylated derivatives of natural substances such as insulin receptors (11). Avidin-conjugated enzymes are used, e.g., in Southern blots to detect biotinylated DNA, in Western blots, and in ELISA (12,13). We have coupled biotin to peptides to detect those with avidin-conjugated peroxidase or alkaline phosphatase after IEF in polyacrylamide gels. The optimal conditions for fixation and staining with avidin-conjugated AP or POD are described under Materials and Methods.

The *N*-hydroxysuccinimide ester of the biotinylation reagent reacts with the free amino or guanidino group of the peptides. However, the reagent displays a reactivity with a limited selectivity toward amino groups as opposed to guanidino groups (of arginine) when the pH is lowered from 8.5 to 7.5 and the molar excess is reduced from 3.5 to 1.5.

Biotinylation of more than one amino group reduces the solubility of the peptide and its susceptibility to glutaraldehyde fixation. In addition, the heterogeneity of the peptide with respect to its biotinyl residues will complicate its use as a substrate to monitor enzymatic reactions. In preliminary experiments (data not shown) the method proved to be particularly useful for the detection of peptides synthesized on a solid-phase resin matrix. Since the N-terminal protective group can be cleaved selectively, the N-terminal amino group can be biotinylated selectively (i.e., while their side chains are still protected) to obtain a monobiotinylated synthetic peptide.

Excess proteins in the reaction mixture can be removed by heat precipitation. Other (unlabeled) peptides do not interfere with the sensitivity. Thus the biotinylated peptide can be detected, for instance, in cell lysates (data not shown) or in heat-precipitated serum.

An average of 1.5 molecules of avidin were coupled to the horseradish POD (14), AP (15), and β-galactosidase (16) used (cf. Materials), bringing the average molecular weight of these enzymes to 142,000, 188,000, and 642,000, respectively (17). Molecules with a molecular weight below 500,000 are freely diffusable in the polyacrylamide gels used in the present study (18). This would explain our observation that avidin-coupled POD and AP stained the biotinylated peptides in the polyacrylamide gels (even if the pore size did shrink somewhat during fixation), whereas avidin-coupled β-galactosidase did not.

The sensitivity limit is below 10 pmol (e.g., for ATII), which represents a 1000-fold improvement over existing IEF techniques for the analysis of low molecular weight peptides. The peptide bands can be scanned and the peak area is proportional to the amount of peptide present over a greater than 100-fold range.

The technique is intended for monitoring enzymatic reactions in which the biotinylated peptides serve as substrates. It can be applied to any peptide which after biotinylation displays a free amino or guanidino group and does not require blotting to nitrocellulose or other matrices. The observation that biotinylation of the peptides results in a shift of the isoelectric point does not interfere with the intended application.

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