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REACTION DETECTOR SYSTEM FOR THE SIMULTANEOUS MONITORING OF PRIMARY AMINO GROUPS AND SULFHYDRYL GROUPS IN PEPTIDES ELUTED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reaction detector system is described which can be used for simultaneous analysis of primary amino groups and sulfhydryl groups in peptides separated by high-performance liquid chromatography. By use of an automatic split valve system, well-defined portions of the effluent are diverted alternately into one of the two reaction detectors, based on the ninhydrin and Ellman assay, respectively. In the latter case the dithioerythritol included in the elution buffers for thiol preservation is eliminated with arsenite. For selective recovery of eluted peptides the amino/thiol group analyser can easily be converted into a high-yield monitoring system compatible with volatile, UV-opaque gradient buffers. In this version an automatic sample valve is used for the thiol assay, removing picomole amounts of material from the effluent destined for collection. In preparative applications there is a progressive net gain in sensitivity with increasing proportion of collected material. Under conditions of optimum recovery *ca.* 90% of the material could be recovered at a total consumption in the two detectors of less than 100 pmol. Experimental conditions are described which would allow for the additional identification of tryptophan, tyrosine and lysine-containing peptides. The monitoring system may also be used without modification for the assessment of key residues in polypeptides and proteins.

INTRODUCTION

In the isolation of peptides from protein hydrolysates by high-performance liquid chromatography (HPLC) it may be useful to combine detectors based on different principles for identifying specific amino acid residues of structural and functional interest. Analytical and preparative techniques at the sub-nanomole level based on absorbance and fluorescence detection of proteolytic fragments containing tyrosine, tryptophan or lysine have been reported[1].

It is of special interest to characterize cysteine-containing peptides from protein hydrolysates, since they are often implicated in the local chemical environment that determines the SH-reactivity in proteins. Pre-column derivatization techniques fre-

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quently used for this purpose involve some uncertainity with respect to the specificity of the reactions[2,3] and the stability of the derivatives during proteolytic digestion and subsequent peptide analysis[4–6]. These difficulties are circumvented by the post-column reaction detector systems described below, in which samples of the column effluent are diverted at regular intervals and destined alternately for primary amino group and sulfhydryl group analysis.

A reaction detector specific for primary amino groups, with an optimum net sensitivity of *ca.* 100 pmol in routine preparative experiments and a recovery of up to 90% of the peptide material, has been described previously[7,8]. By use of a stream splitting valve, small fractions of the column effluent were intermittently diverted toward a ninhydrin reactor and photometer. This system could be combined with a second stream splitting valve adapted for sulfhydryl analysis by a modified version of the dithioerythritol (DTE)/sodium arsenite/5′,5-dithiobis(2-nitrobenzoic acid) (DTNB) method of Zahler and Cleland[9]. The same method has been adapted earlier for the detection of disulfide-linked peptides in column effluents at the 1-μmol level[10]. In the applications described below, DTE is added in advance to the buffers for protection of the thiol functions during elution[11]. After amino group monitoring, the effluent and reagents are directed toward the sulfhydryl reaction detectors. The detector functions involve DTE elimination, DTNB reactions and colorimetric assay. For preparative applications, the monitor for primary amino groups is combined with a stream sampling valve commonly used for fluorescamine detection[12,13]. By this valve, small volumes of the column effluent are transferred into a transport solution continuously passed to the thiol reaction detector. In the integrated monitoring system less than 90 and 140 pmol of model peptide (glutathione) suffice for the analysis of amino and sulfhydryl groups, respectively. Under the conditions of optimum recovery *ca.* 90% of the eluate can be collected at a total net detector sensitivity of less than 100 pmol.

EXPERIMENTAL

Reagents

Ninhydrin reagent and elution buffers (A, 0.1 *M* pyridine acetate, pH 3.5; B, 1 *M* pyridine acetate, pH 5; C, 2 *M* pyridine acetate, pH 5) were as described previously[7,8], but the eluents contained 0.04 m*M* DTE. Neutralizer (solution D) for post-column pH-adjustment and DTE elimination contained 3M tris-(hydroxymethyl)aminomethane (Tris), 7.5 m*M* sodium arsenite and 40 m*M* ethylenediaminetetra-acetate (EDTA). The 0.25 m*M* DTNB solution (solution E) contained 20 m*M* sodium acetate buffer, pH 5. Tris, DTE, DTNB and glutathione (reduced form) were from Sigma (St. Louis, MO, U.S.A.). All chemicals were of reagent grade. Solutions were made up in water passed through a deionizer, Milli-Q Reagent Grade Water System (Millipore, Bedford, MA, U.S.A.) filtered and thoroughly deaerated.

Sample preparations

Bovine brain S-100 was prepared and characterized as described previously[14]. Peptic digestion of the denatured S-100 was for 16 h (37°C) in 10 m*M* hydrochloric acid–1 m*M* DTE (enzyme-to-substrate ratio, 1:50). After lyophilization, peptides were dissolved in chromatographic sample buffer (0.3 *M* pyridine acetate, pH 2.5).

Aliquots of the hydrolysates were standardized by acid hydrolysis and microanalysis of basic amino acids. Glutathione, used as model peptide, was dissolved in water and standardized by DTNB assay. Aliquots were mixed with sample buffer and 10–20-μl samples used for analysis.

Chromatographic system

The instrumentation was basically as described previously[7,8]. Aliquots of peptic hydrolysates were applied to a 250 × 2 mm I.D. column of Aminex A-5 resin (particle size 13 μm) (Bio-Rad, Richmond, CA, U.S.A.) and eluted with two consecutive gradients of pyridine acetate. In the first gradient, buffer B was directed into the 50-ml gradient mixer, filled with buffer A. After 6 h, buffer C was passed into the mixer. In the experiments with the dual monitoring system, a 150 × 2 mm I.D. column of Aminex-A8 resin (particle size 7 μm) was used for stepwise or gradient elution of the model peptide. The columns were maintained at 52°C by use of a solid thermoregulator. A reciprocating piston pump (Minipump Duplex, Milton Roy) was used for both the eluent and the ninhydrin reagent. Ultralinear displacement pumps (Labotron, Gelting) were used for delivery of transport solution (identical with buffer B) and of solutions D and E. Absorbancies at 570 and 420 nm were measured in a Labotron UDC photometer provided with two 10-μl flow cells (10-mm light path) and were recorded with a three-channel strip-chart recorder (Model B-316, Rikadenki Koyo, Tokyo, Japan).

Post-column reaction detectors

Slider valves, pneumatic actuators, solenoids and reactors (0.8 mm I.D., Teflon, dual analytical system, Fig. 1A) were obtained from Altex, Berkeley, CA, U.S.A. PTFE tubing adapters, post-column tubing and reactors (0.3 mm I.D., PTFE, dual monitoring system, Fig. 1B) were from Durrum, Palo Alto, CA, U.S.A.

Dual detector analytical system. As shown in Fig. 1A, both detectors include a stream splitting valve consisting of two three-way slider valves. They are operated by oppositely acting pneumatic actuators controlled by the reciprocating solenoids S1, S2 (valve unit I) and S3, S4 (valve unit II). Impulses of optional duration are given to the solenoids by an electronic timer. The combined valve units operate in repetitive, two-phase switching cycles, exemplified by the 60-sec cycle used in the experiments (Fig. 2).

Phase 1: Solenoids S2 and S4 are actuated simultaneously for 30 sec. During this period eluate and ninhydrin reagent are directed by valve unit I toward the ninhydrin reactor R1 (10-min delay), while solutions D and E are recycled (solid lines in Fig. 1A).

Phase 2: Solenoids S1 and S3 are actuated simultaneously for 30 sec. Ninhydrin reagent is now recycled (RC). Eluate and solution D pass via the mixing tee toward reactor R2 for DTE elimination (5-min delay). Eluted thiols are subsequently allowed to react with DTNB in reactor R3 (2-min delay) by the simultaneous introduction of solution E into the second mixing tee (dashed lines in Fig. 1A).

Dual detector monitoring system. As shown in Fig. 1B, the amino group reaction detector includes a dual stream-splitting valve (valve unit I) identical with that described for the analytical system. It is operated by two oppositely acting actuators controlled by the reciprocating solenoids S1, S2. The sulfhydryl group reaction de-

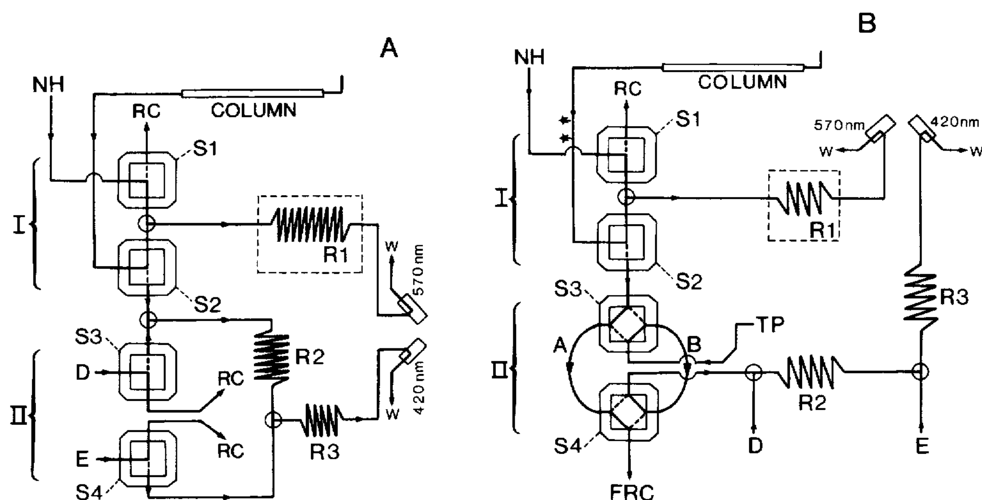

Fig. 1. Flow diagrams of the amino/thiol group analysing and amino/thiol group monitoring systems. (A) Amino/thiol group analyser. I = Valve unit I (stream-splitting valve) operated by two oppositely acting pneumatic actuators controlled by solenoids S1, S2. II = Valve unit II, operated like I and controlled by solenoids S3, S4. The two stream-splitting valve units operate continuously in two-phase cycles of optional length, as determined by timer-preset, synchronous on/off signals to the solenoids. Phase I (amino group assay): S2 + S4 on/S1 + S3 off. Phase 2 (thiol group assay): S1 + S3 on/S2 + S4 off. In the experiments shown in Fig. 2. (60-sec cycles), the signals were given at zero and 30 sec, respectively. Solid lines in valve units indicate flow paths of effluent and reagents during phase 1, dashed lines alternative flow paths during phase 2. R1 = ninhydrin reactor; R2 and R3 = reactors for DTE elimination and DTNB reaction, respectively; NH = ninhydrin reagent; D = Tris-arsenite-EDTA solution; E = DTNB solution; RC = recycling of reagents during idle phase; W = waste. (B) Amino/thiol group monitor. I = Valve unit I operated by two oppositely acting pneumatic actuators controlled by solenoids S1, S2. II = Valve unit II (stream-sampling valve) operated like I and controlled by solenoids S3, S4. The combined valve units operate continuously in three-phase switching cycles of optional length as determined by timer-preset, synchronous on/off signals to the solenoids. Phase 1 (amino group analysis): S2 on/S1 + S4 off. Phase 2 (thiol group analysis and collection): S1 + S3 on/S2 off. Phase 3 (thiol group analysis and collection): S4 on/S3 off. In the experiments shown in Fig. 3 (21-sec cycles) the signals were given at zero, 3 and 12 sec, respectively. Dashed lines indicate alternative flow paths of effluent and transport solution during phases 2 and 3 of the switching cycle, when the effluent is passed toward valve unit II and fraction collector. A/B = sample loops; TP = transport solution (buffer B). Asterisks indicate proposed sites of insertion of UV and native fluorescence detectors in analytical and preparative applications.

tector includes a dual stream-sampling valve (valve unit II) consisting of two four-way slider valves connected by two identical sample loops (A, B) of optional length. It is operated like valve unit I by the reciprocating solenoids S3, S4. The automatic dual monitoring of the effluent is controlled by an electronic timer, actuating the solenoids for short, optional time periods synchronous with the de-actuation of the oppositely acting solenoids. The combined valve units operate in repetitive, three-phase switching cycles, exemplified by the 21-sec cycle, used in the experiments (Fig. 3).

Phase 1: Solenoid S2 is actuated for 3 sec. During this period eluate and ninhydrin reagent are directed by valve unit I via the mixing tee toward reactor R1 (2.75-m coil, 4-min delay) and the 570-nm photometer unit. In the temporarily disconnected valve unit II transport solution (TP) flows through sample loop B toward the thiol reactor and the 420-nm photometer unit. The eluent flow through sample loop A toward the collector (FRC) is arrested (solid lines in Fig. 1B).

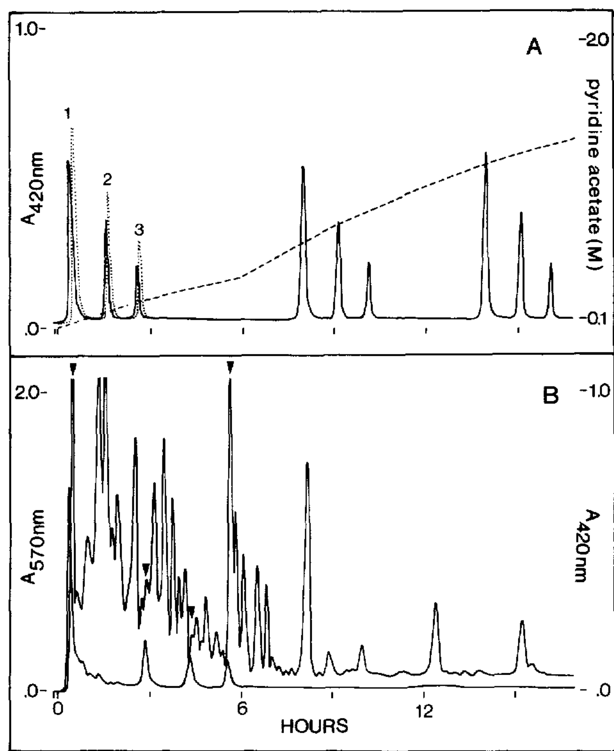

Fig. 2. Functional characteristics of amino/thiol group analyser. Peptides were eluted from a 250 × 2 mm I.D. column of Aminex A-5 cation-exchange resin (52°C) with two consecutive gradients, ranging from 0.1 to 0.44 and from 0.44 to 1.35 *M* pyridine acetate. Eluent and ninhydrin flow-rates were 4 ml/h. Solutions D and E were delivered at 4 ml/h and 1 ml/h, respectively. With switching cycles of 30 + 30 sec, equal 33-μl portions of the effluents were diverted alternately for thiol group and amino group analysis. (A) At different buffer concentrations following amounts of the model peptide (glutathione) were injected: 1 = 48 nmol; 2 = 24 nmol; 3 = 12 nmol. Solid line, absorbance at 420 nm; dotted line, absorbance at 570 nm (included only for the first three assays). (B) Dual analysis of a peptic hydrolysate (50 nmol) of acidic brain protein S-100. Upper curve, absorbance at 570 nm; lower curve, absorbance at 420 nm. Arrowheads indicate sulfhydryl-containing fragments recorded in the lower curve.

Phase 2: Solenoids S1 and S3 are actuated simultaneously, synchronous with the de-actuation of solenoid S2. Ninhydrin reagent is now recycled (RC). Eluate passes through the connected valve units via sample loop B toward the collector. At the same time the content of sample loop A is passed into the transport solution (dashed lines in Fig. 1B). DTE elimination and reaction of the eluted thiols with DTNB are performed sequentially for 2 min in reactors R2 (8.8-m coil) and R3 (10-m coil) by the continuous infusion of solutions D and E.

Phase 3: Solenoid S4 is actuated 9 sec after the start of phase 2, synchronous with the de-actuation of solenoid S3. The eluate is still directed toward the collector, but now via sample loop A. The effluent momentarily retained in sample loop B is now displaced by the transport solution and passed toward the thiol detector (solid lines, valve unit II). From this position a new switching cycle is started by the de-actuation of solenoids S1 and S4 and the synchronous actuation of solenoid S2.

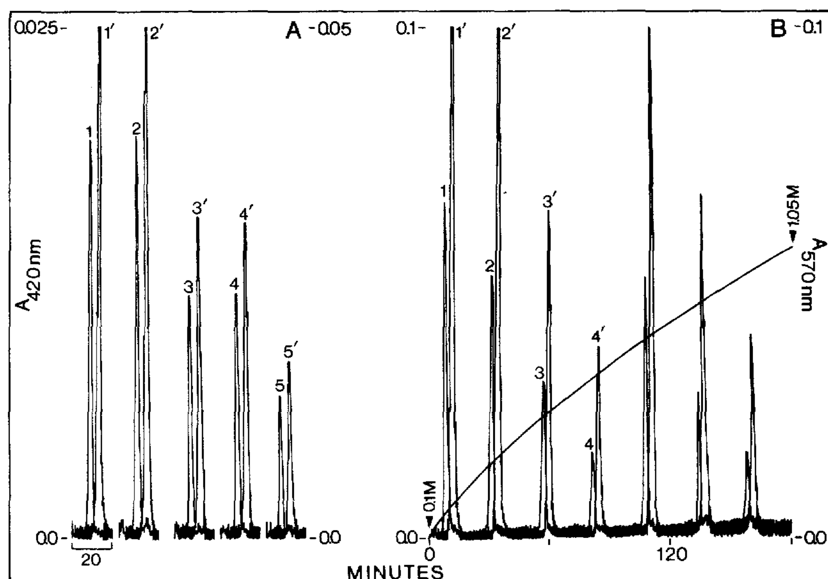

Fig. 3. Performance of integrated amino/thiol group monitor under different conditions of elution. Flow-rates and sample loop volumes were as in Fig. 4. Ninhydrin and eluent were delivered at equal flow-rates. Switching cycles were 3 + 9 + 9 sec. In (A) equilibration buffer was used as eluent, in (B) a 0.10–1.05 *M* pyridine acetate gradient, as indicated. The amounts of glutathione injected were: (A) 2 × 2.5 nmol, 2 × 1.25 nmol, 625 pmol; (B) 7.5 nmol, 2 × 5 nmol, 2 × 2.5 nmol, 2 × 1.25 nmol (serial additions). Full-scale deflection at 570 nm corresponded to 0.05 (A) and 0.10 (B) absorbance units, and at 420 nm to 0.025 (A) and 0.10 (B) absorbance units. Of the additions the amino group detector (570 nm, peaks 1'–5') consumed 14%, the thiol group detector (420 nm, peaks 1–5) 22%, and net recovery of collected material was 64%. Thus, peaks 5' (amino group assay) and 5 (thiol group assay) in experiment A represent 88 and 137 pmol of peptide, respectively.

Flow-rates

In the experiments using the dual analytical system (Fig. 1A) the eluent and ninhydrin reagent were pumped at a flow-rate of 4 ml/h. Solutions D and E were delivered at flow-rates of 4 ml/h and 1 ml/h, respectively. In the experiments with the dual monitoring system eluent and ninhydrin reagents were pumped at a flow-rate of 12 ml/h. Transport solution and solution D were delivered at 10.6 ml/h and solution E at 3 ml/h.

RESULTS AND DISCUSSION

The dual detector systems described utilize the principles of stream splitting and stream sampling of column effluents, which provides great versatility in the choice of experimental conditions. Thus, the systems can easily be combined with UV-opaque eluents, frequently used as effective, volatile peptide solvents in ion-exchange[8,15] and reversed-phase HPLC[16,17]. This is demonstrated by the experiments described below, in which proteolytic and model peptides were eluted with pyridine acetate buffers from cation-exchange resins.

Amino/thiol group analyser

In the experiments described in Fig. 2A gradients ranging from 0.1 to 1.4 *M* pyridine acetate were used for the repetitive elution of a model peptide (glutathione). Stable baselines were obtained, and thiol peaks were recorded with good linearity of the detector response. The final pH values of the reaction mixtures were close to 8, showing that the neutralizer afforded adequate pH control for the DTNB reaction. Under the same conditions, peptic peptides from S-100 protein were eluted from the column and assayed by the amino/thiol group analyser. The dual recorder tracings obtained are shown in Fig. 2B. One major and three minor thiol peaks could be observed in the chromatogram, evidently representing the sulfhydryl-containing sequences of the protein[14]. The system can easily be adapted for higher flow-rates and optimized for higher sensitivity of detection by using reactors with smaller tube diameter and increasing the scale expansion of the recorder. However, for the selective recovery of intact thiol peptides suitable for sequence studies the system has to be modified by substituting a stream-sampling valve for the stream-splitting valve in the thiol assay (Fig. 1B). As will be discussed below the modified system retains the purely analytical functions and may also be adapted to other derivatization techniques.

Amino/thiol group monitor

The monitoring system was primarily designed for routine preparative experiments, but the detectors can also be used, separately or integrated, in purely analytical applications with only minor modifications.

Analytical applications. For the analysis of primary amino groups alone the valve units are arrested in phase 1, thus directing effluent and ninhydrin continuously into a 20-m reactor coil to give a 4-min heating time[8]. For the analysis of sulfhydryl groups the valve units are positioned as described for phase 2, but with the transport solution disconnected. The effluent is passed directly from valve unit II into the mixing tee (Fig. 1B). Fig. 4A shows this application with glutathione as model peptide. Thiol peaks were recorded with high reproducibility and good linearity of the detector response. Full-scale deflection (0.025 a.u.f.s.) was obtained with 800 pmol, and 156 pmol gave satisfactory peak heights at a signal-to-noise ratio of 7. The lower limit of detection was *ca.* 70 pmol. The sensitivity of the detector could be increased several-fold by using microbore columns in conjunction with the standard 20-mm flow-cell of the photometer.

Preparative applications. For sulfhydryl group monitoring alone, valve unit I is positioned as described for phase 2, but with water recycled in the ninhydrin line. Only the sampling valve (valve unit II) is coupled to the timer. From the effluent, passed toward the collector, samples are removed at regular intervals (9 sec) for thiol detection. In analogy with what has been shown previously for the stream-splitting device[8], this arrangement provides for a precise quantification of the eluted components, since their concentrations in the assay are basically unaffected by the sampling. With the rapidly eluted model peptide, shown in Fig. 4B, there was still a net gain in sensitivity, considering that 78% of the material was collected. Because of the relatively high sampling frequency used, recorder tracings were continuous. The reproducibility of the system and the linearity of the detector response were as shown for the analytical application.

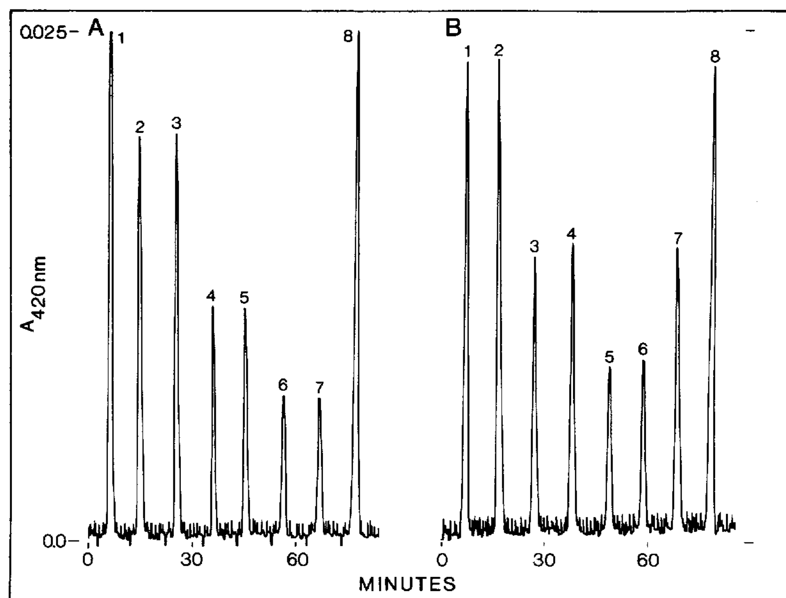

Fig. 4. Performance of the thiol group detector in analytical and preparative applications. A 150 × 2 mm I.D. column of Aminex A-8 cation-exchange resin (52°C) was used. The eluent was 0.1 *M* pyridine acetate, pH 3.5, containing 0.04 m*M* DTE, and delivered at a flow-rate of 12 ml/h. Flow-rates of solutions D and E were 10.6 and 3 ml/h, respectively. Full-scale deflection at 420 nm corresponded to 0.025 absorbance units. (A) Valve units arrested in phase 2 (Fig. 1B, dashed lines) with transport solution disconnected. The following amounts of glutathione were injected consecutively: 1.25 nmol (peak 1); 625 pmol (peaks 2, 3); 312 pmol (peaks 4, 5); 156 pmol (peaks 6, 7) and 800 pmol (peak 8). (B) Experimental conditions were as in A, but with valve unit II in operation and transport solution delivered at the (non-optimal) rate of 10.6 ml/h. The sampled volumes (6.6 μl) were transferred into the detection stream at 9-sec intervals. The rest of the effluent was passed into the fraction collector. The following amounts of glutathione were injected: 2.5 nmol (peaks 1, 2, 8); 1.25 nmol (peaks 3, 4, 7) and 625 pmol (peaks 5, 6). Of these additions 550, 275 and 137 pmol were consumed for detection, and net recovery was 78% of the material.

Integrated monitor performance. Fig. 3A illustrates the dual monitoring of different amounts of glutathione with a switching cycle of 3 + 9 + 9 sec (Fig. 1B). Compared with the preceding experiment, there was a slight decrease in the sensitivity of the thiol reaction detector because additional transport solution passed through valve unit II during the ninhydrin assay (phase 1). At the lowest sample load (625 pmol) peaks were still satisfactorily recorded by the two detectors, whereas *ca.* 400 pmol (*ca.* 64%) were recovered in the fraction collector.

The integrated valve system may also be used for purely analytical applications. In this version the entire fraction of the effluent passed toward valve unit II is used for detection. The eluent and the transport solution have to be delivered at equal flow-rates. A switching cycle of 4 + 2 + 2 sec is suggested. It has been shown previously in amino group analysis alone[8] that the loss in peak heights is very moderate at this high sampling frequency. As discussed for the preparative applications, there is also a net gain in sensitivity under these conditions.

Gradients. The experiments described above were repeated with buffers A, B and C, introduced stepwise into the column. The final pH values of the reaction

mixtures destined for the thiol assay were 8.3, 8.4 and 7.9, respectively, providing for an almost identical detector response (not illustrated). In analogy with the experiment described in Fig. 2A gradient elution of the model peptide, repetitively injected into the column, gave stable base lines with almost indistinguishable top heights (Fig. 3B). Thus, the system can be used to monitor preparative microscale separations of protein hydrolysates requiring gradient elution.

Optimizing the monitor performance. The sensitivity of the monitoring system, as illustrated above, can be increased by the following modifications: In the amino group assay the ninhydrin reaction can be performed at higher temperature (134–140°C), reducing the reaction time to *ca.* 100 sec[18]. In the case of the thiol assay, proper adjustment of the transport solution minimizes dilution of the aliquot destined for analysis. At the eluent flow-rate specified in Figs. 3 and 4, a reduction of the flow-rate of the transport solution from 10.6 to 2.65 ml/h still affords complete displacement of the sample loop content. The mixing with neutralizer and DTNB solutions, concomitantly adjusted to proportionally lower flow-rates, is not impaired. Band broadening in coiled tubular reactors is a function of tube diameter and the reaction time[19]. Since the DTNB reaction is nearly complete within 30 sec, an additional increase in sensitivity is obtained by using a proportionally shorter reactor, thus minimizing diffusion of the eluted material.

The net recovery of collected material can be increased by (a) using smaller sample volumes, and (b) increasing the sampling intervals. Thus, with 6.6-μl sample volumes consumed in each assay at the switching cycle of 2 + 18 + 18 sec, *ca.* 90% of the effluent is recovered at a total net sensitivity of the detector systems of *ca.* 100 pmol. Optimum recovery should be particular useful in preparative separations of small amounts of protein hydrolysates[20]. The predictable loss in peak height of the early eluted peptides in this application would be of minor consequence, since the accuracy improves progressively in the course of the elution (*cf.* ref. 8).

Further applications. Reversed-phase HPLC with UV-transparent buffers is frequently used for peptide preparation[21,22]. UV monitoring of the effluents and subsequent native fluorescence detection allow the identification and discrimination of tryptophan- and tyrosine-containing peptides[1]. Combining these detection principles with the monitoring system described above would provide information on two additional key residues. The UV and fluorescence detectors, in series, should be inserted as indicated by the asterisks in Fig. 1B. In addition, the neutralizer (solution D) has to be adapted to the reversed-phase chromatographic solvent system used. With conventional gradients of acetonitrile–0.05% trifluoroacetic acid, 0.2 *M* Tris–HCl (pH 8.4) affords accurate pH control for the DTNB rection (unpublished results). In this version the stream-splitting valve (valve unit I) may be omitted or used for alternative analysis, as described below.

Since the integrated valve units described in Fig. 1B provide for three separate flow streams, which can be adjusted independently, other derivatization techniques may be used alternatively. For instance, by replacing ninhydrin with *o*-phthalaldehyde reagent the stream-splitting valve can be used for the identification of lysine-containing peptides[1]. In combination with on-line UV and native fluorescence detectors, the integrated monitoring system, in this version, would provide for the identification and recovery of peptides containing four different specified amino acid residues of structural and functional importance. The use of 4-fluoro-7-nitrobenzo-

2,1,3-oxadiazole (NBD-F) for post-column fluorometric detection of primary and secondary amines has recently been reported. Under acidic conditions, NBD-F reacts with thiols[23]. The amino/thiol group monitor described above would seem very suitable for this highly sensitive assay. The stream-splitting valve would be used for thiol detection by mixing the diverted effluent with acidic buffer (*e.g.* 4 *M* sodium acetate, pH 4.5), and subsequently by an additional, synchronously operated stream-splitting valve, with reagent and baseline suppressor (hydrochloric acid). The stream-sampling valve would be used for monitoring primary/secondary amines by successively introducing borate buffer, NBD-F reagent and baseline suppressor into the detector line.

Although this report is focused on peptide identification, the methodology described should prove valuable for assessment of key residues in larger macromolecules, such as CNBr fragments or proteins in conjunction with the above or other separation procedures. Owing to its great flexibility it should also be useful as an instrument for process control.

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