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NOVEL APPROACH TO RAPID AND SENSITIVE LOCALIZATION OF PROTEIN DISULFIDE BRIDGES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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SUMMARY

No method has yet been developed for the chemical localization of a disulfide linkage which is capable of using the very low amounts of proteins or polypeptides often encountered today. The novel approach reported herein is entirely based on the use of high-performance liquid chromatography; hence it offers the rapidity, reliability and sensitivity necessary in order to solve this most important problem. The first step involves the separation of a proteolytic digest of the native protein by reversed-phase chromatography. The separation is monitored using a UV spectrophotometer coupled to an electrochemical detector able to detect *solely* disulfide-containing peptides. The second step involves the chemical reduction and alkylation of these peptides followed by purification under the same conditions as the initial run. Amino acid composition allows identification of each peptide and its precise localization in the protein sequence. This report is meant to demonstrate the utility of the electrochemical detector for detecting disulfide-containing peptides and to validate the feasibility of this proposed approach, by using as a model a 26-amino acid peptide atrial natriuretic factor containing one disulfide bridge.

INTRODUCTION

In recent years, high-performance liquid chromatography (HPLC) has been instrumental in unraveling protein and peptide structures using minimal amounts of materials. Whereas the means to achieve isolation, purification and chemical analysis such as amino acid composition and sequencing have all benefited from the improvements made in HPLC technologies, the localization of disulfide bridges in a protein, which is often the last endeavor to be made in sequence analysis, has received little attention. This aspect can be related either to the lack of suitable methodologies or to the absence of specific on-line detection methods for disulfide-containing peptides.

Indeed, some methods such as random acid hydrolysis in presence of thiols[1] or the well-known diagonal paper electrophoresis technique[2] clearly lack the convenience or sensitivity needed today. Others, such as chemical synthesis of the various conformers[3] or the partial reduction of disulfide bridges[4], require prior knowledge

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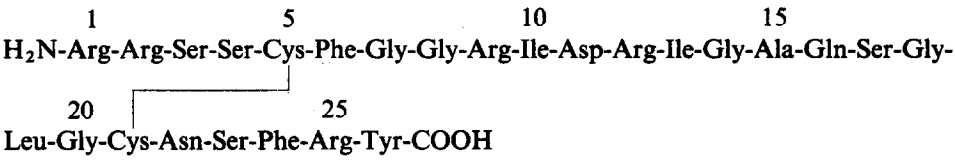
of the complete sequence and are very tedious. Moreover, the difficulties encountered in resolving the arrangement of disulfide linkages have led to an increasing number of bridges being inferred from theoretical considerations based on amino acid sequence homologies, structure-activity relationships etc.; examples can be found in the case of the γ-subunit of nerve growth factor[5] in view of its relatedness to serine protease such as trypsin or the recently proposed β_2-inhibin moiety[6]. Furthermore, in order to chemically determine the arrangement of disulfide bridges, it is deemed necessary to rely on a sensitive and specific method to detect the disulfide-containing peptides in a sometimes extremely complex mixture yielding multiple chromatographic fractions. Numerous methods to detect disulfide bonds in polypeptides and proteins have been proposed as discussed by Thannhauser *et al.*[7]. The detection of disulfide groups can be accomplished by direct analysis of sulfur as proposed by Fowler and Robins[8] or, in most methods discussed in ref. 7, by either oxidation or reduction of the disulfide into more easily detectable entities, such as thiols, which can be detected by various chromogenic reagents. Unfortunately, most of these methods even though specific and sensitive cannot be adapted to on-line detection after a chromatographic run, rendering the whole process very long. One possible solution, as described by Studebaker *et al.*[9], is to rely on the use of a polymer-bound thiol for detecting disulfides as a post-column detector. Another possible solution has been recently proposed by Allison and Shoup[10] who demonstrate the feasibility of detecting both free thiols and disulfides, when using glutathione as a model, by electrochemical detection (ED).

Thus, it is the aim of this report to demonstrate the feasibility of using such a detector in the elucidation of the arrangement of disulfide bridges in proteins and peptides. The proposed approach, schematized in Fig. 1, employs the electrochemical detector to detect solely the disulfide bridge-containing peptides released from an enzymatically produced (preferentially at acid pH to minimize disulfide interchange) digest of the native starting material. Following their detection, these peptides are collected, chemically reduced and alkylated and subsequently rechromatographed under the same conditions as the original separation. The resulting peptides, separated from unmodified peptides that could have contaminated them in the first separation, are finally identified by amino acid composition or sequence analysis using classical methods. The basic question is thus whether the electrochemical detector can specifically detect these peptides and, if so, can the whole scheme be carried out. In order to answer these problems, the present report will describe results obtained with different model peptides, such as glutathione, [Arg[8]]-vasopressin and oxytocin; it will also discuss the validity of the proposed approach with reference to synthetic rat atrial natriuretic factor (ANF) as a model, which has the folllowing sequence:



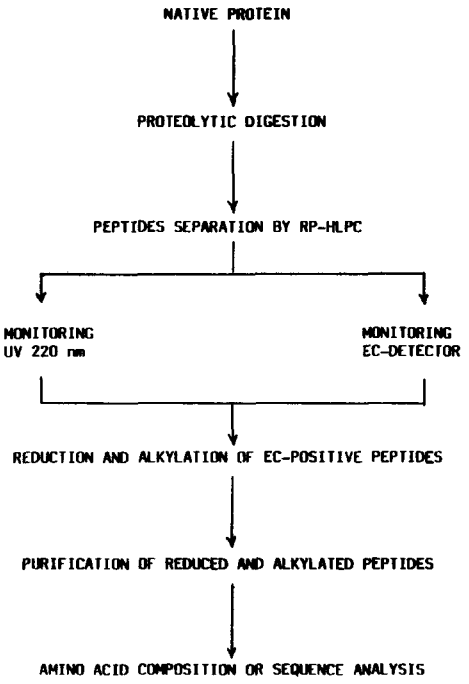


Fig. 1. Proposed scheme for the determination of disulfide bridges in protein.

EXPERIMENTAL

Materials

Glutathione (reduced state) was purchased from Sigma, whereas synthetic oxytocin and [Arg^8]-vasopressin were obtained from Ferring (Läkemedel, Sweden). Synthetic rat atrial natriuretic factor (ANF 8–33) was chemically synthesized by Merck, Sharp and Dohme (West Point) according to ref. 11.

Guanidine hydrochloride was of ultra-pure grade (Schwarz/Mann), whereas dithiothreitol (DTT) and iodoacetic acid were obtained from Sigma and Eastman Kodak, respectively. DCC-treated trypsin was obtained from Miles-Seravac and dissolved in 2m*M* hydrochloric acid before using it at a concentration of 5 mg/ml.

Acetonitrile (Caledon) was of HPLC-grade quality, and trifluoroacetic acid (TFA) from Sigma was redistilled before use; all HPLC solvents were filtered through 0.2-μm filters, degassed under vacuum and exhaustively purged of oxygen by bubbling helium gas through them. HPLC solvents were further kept in closed reservoirs continuously maintained under He atmosphere. All amino acid analysis reagents and solvents were obtained from Beckman.

Apparatus

Reversed-phase HPLC was carried out on a μBondapak C_{18} column (30 cm $\times$ 3.9 mm I.D., Waters). A Varian Model 5500 liquid chromatograph linked to a Vista 402 (Varian) plotter/integrator was used for all experiments: injections were

carried out through a pneumatically activited injector (Valco) equipped with a 50-μl or 200-μl loop.

The effluent was monitored at 220 nm by a variable-wavelength UV-200 detector (Varian). When electrochemical detection was used, it was coupled to the outlet of the UV detector and the chromatograms were recorded simultaneously. The electrochemical detector consisted of dual LC-4B (BAS) amperometric controllers linked to a detector cell (BAS) containing two mercury/gold electrodes in series (and prepared according to the manufacturer's specifications); the thin-layer channel was defined by two TG-5M PTFE gaskets (BAS). The silver/silver chloride reference electrode (RE-1, BAS) was positioned downstream of the detector cell. Monitoring of the electrochemical-chromatogram was accomplished with a remote recorder Pharmacia REC-482.

Amino acid analyses were carried out on a modified Beckman 120C amino acid analyzer equipped with a Model 126 computing integrator. The amino acids are separated with a single column containing Dionex DC5A resin.

Reversed-phase HPLC

All peptides used were dissolved at a concentration of 1 μg/μl in 0.1% TFA, and appropriate aliquots were injected. The solvents used for elution were obtained by mixing an aqueous solution of 0.1% TFA and an organic phase composed of 0.1% TFA in water: acetonitrile (10:90); the reversed-phase column was operated at 1.0 ml/min. When the injected material needed to be recovered, the peptide-containing fractions as judged by UV absorption were manually collected at the outlet of the UV detector. For ED, the upstream electrode was held at an applied voltage of -1.0 V (*vs.* Ag/AgCl) whereas the downstream electrode was held at $+0.2$ V (*vs.* Ag/AgCl) unless otherwise stated.

Trypsin digestion of synthetic rat ANF

Synthetic rat ANF (100 μg) was dissolved in 0.1 *M* ammonium bicarbonate to which was added a suitable amount of a freshly prepared solution of DCC-treated trypsin in 2 m*M* hydrochloric acid to yield an enzyme:substrate ratio of 1:40. The digestion was conducted at 37°C, and aliquots (5–10 μl out of 100 μl digest) were taken out at 0, 120, 210 and 330 min; these aliquots were immediately analysed by reversed-phase HPLC after they had been dried under nitrogen and dissolved in 25 or 50 μl 0.1% TFA prior to injection. After the digestion, the remainder of the digest was diluted five-fold with 0.1% TFA, and three separate runs (100-μl injection) were made in order to collect the released peptides, based on the UV and electrochemical chromatograms.

Reduction and alkylation of the electrochemically positive peptides

Each of the electrochemically positive fractions was dried under vacuum and reconstituted in 100 μl of freshly prepared 0.5 *M* Tris–HCl buffer (pH 8.4) containing 6 *M* guanidine hydrochloride and 1 m*M* EDTA. The solution was then made 10 m*M* in DTT by adding 15 μl of a freshly prepared DTT solution (10 mg/ml in the above buffer) and was incubated at 37°C for 1 h. Finally, 30 μl of a freshly prepared iodoacetic acid solution (10 mg/ml in the above buffer) was added and the resulting solution was incubated for a further 30 min at 37°C before it was injected directly on the HPLC column without any prior desalting.

Amino acid analysis

After each fraction had been dried under vacuum, the peptides were dissolved in 400 µl of 5.7 *N* hydrochloric acid containing 0.01% β-mercaptoethanol and a trace of phenol. The evacuated tubes were sealed under vacuum and kept at 108°C for 24 h. The dried-down hydrolysate was reconstituted with 150 µl of citrate buffer (pH 2.2) before analysis. Quantitation of amino acids was obtained using an internal standard of 2.5 nmole norleucine.

RESULTS AND DISCUSSION

Application of the electrochemical detector to disulfide-containing peptides

The use of ED depends entirely on the presence of an electroactive group(s) in the substance to be detected. Recently, it has been shown that, in polypeptides for example, they can be detected by their free NH_2 group[12], by the presence of the phenol group of tyrosine[13,14] or the indole group of tryptophan[14] and by modifying them with electroactive substituents such as 3,6-dinitrophthalic anhydride[15]. On the other hand, as discussed by Allison and Shoup[10], disulfide-containing peptides can also be detected by using two mercury/gold electrodes in which the first one (upstream) is used as a generator to reduce the disulfide bridge:

$$RS\text{-}SR + 2H^+ + 2E^- \rightarrow 2RSH \ (E_1 \text{ volts})$$

and the second one (downstream) is used to detect the thiol produced.

$$2RSH + Hg \rightarrow Hg(RS)_2 + 2H^+ + 2E^- \ (E_2 \text{ volts})$$

Thus, whereas free thiol will always be detected, the disulfide-containing peptides can be detected only after being reduced at the first electrode; this in turn offers a way to verify the specificity of the detection.

Initial studies (data not shown) conducted using glutathione, [Arg^8]-vasopressin and oxytocin, revealed that it was indeed possible to detect these peptides specifically with a reversed-phase column and gradient elution with acetonitrile. The results obtained after injecting *ca.* 500 ng of these peptides, while not optimized in terms of sensitivity or quantitation, revealed that the detector response was sensitive to the voltage applied at the downstream electrode (E_2) for a given voltage applied to the upstream one (−1.0 V). Thus, it was optimal, as reported in ref. 10, for glutathione at +150 mV whereas for vasopressin and oxytocin it was closer to +100 mV; it is worth mentioning, at this time, the extreme sensitivity of these electrodes upon the buffer used, their age and also their environment. Nevertheless as shown in Fig. 2, it is possible to detect specifically the presence of a disulfide bridge in the case of [Arg^8]-vasopressin eluting at *ca.* 25.5 min and synthetic ANF eluting at *ca.* 32 min using a gradient of 0.1% TFA and acetonitrile. Thus, at a voltage of −1.5 V (identical results are obtained with −1.0 V) and +0.2 V at the upstream and downstream electrodes, respectively, one can see in the electrochemical chromatogram the peaks corresponding to both of these peptides and also two peaks corresponding to reduced glutathione (*ca.* 4 min) and probably an oxidized form of the same (eluting at *ca.* 5 min). The specificity of the detection is exemplified when the

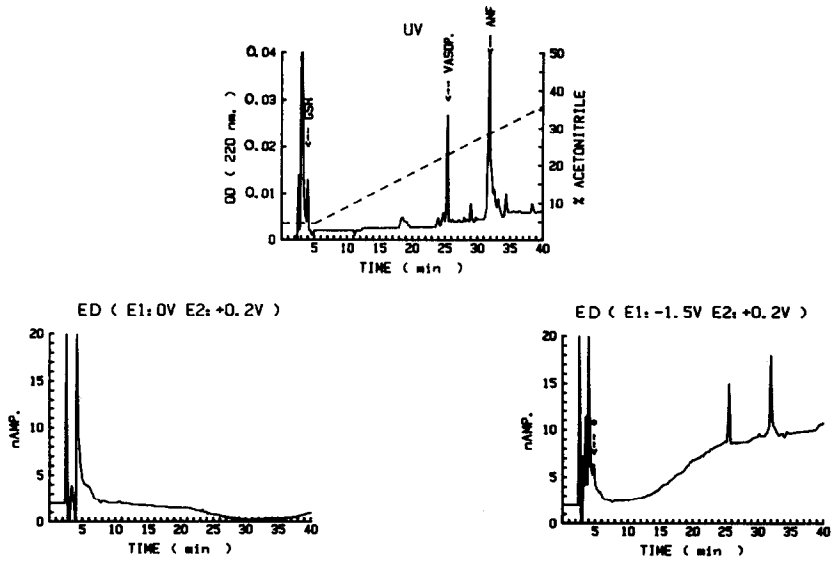


Fig. 2. Reversed-phase HPLC of a mixture containing glutathione, [Arg^8]-vasopressin and synthetic rat ANF monitored using UV absorbance at 220 nm and electrochemical detection with the applied voltage (E_1 and E_2) indicated. The presence of some oxidized form of glutatione is indicated by the asterisk. The acetonitrile gradient used is indicated by the dashed line.

upstream electrode is switched off (E_1 = 0 V), whereupon a single peak is detected corresponding to the free-thiol bearing glutathione; indeed, no peaks are detected for [Arg^8]-vasopressin, synthetic ANF and the possible oxidized glutathione. Moreover, when reduced and alkylated ANF was injected with both electrodes operative, an extremely small peak was observed at the elution position of reduced and alkylated ANF which could have arisen from the incomplete chemical reaction since, with the gradient used, both ANF and reduced and alkylated ANF were not entirely resolved. Similar results were recently presented by Garvie *et al.*[16] who demonstrated the feasibility of detecting vasopressin and analogues at the very low picomole level with the electrochemical detector.

Determination of disulfide bridge in a model peptide

The next step was thus to examine whether it would be possible to detect the peptide resulting from a proteolytic digest that would contain the Cys-5 and the Cys-21 in the synthetic ANF molecule. To this end, trypsin digestion, even though a pepsin digestion is favored considering the lower pH, was chosen because of the resistance of synthetic ANF to the latter enzyme. Using UV absorbance and the electrochemical detector, it was possible to follow the formation of disulfide-containing products with respect to time (data not shown) by injecting *ca.* 5 μg of the digest onto the column. The last aliquot, corresponding to 5.5-h digestion, when examined by both detection methods produced the chromatogram depicted in Fig. 3. Thus, based on UV absorbance, six main peaks can be detected whereas, using the electrochemical chromatogram, only three of these (peaks 3, 4 and 5) should contain disulfide bridges. What remained from the digest (*ca.* 50 μg) was chromato-

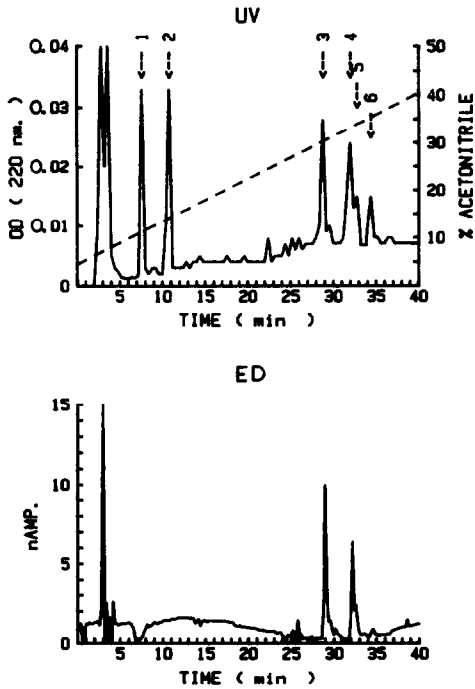


Fig. 3. Reversed-phase HPLC of the synthetic ANF digested with trypsin for 5.5 h monitored by UV absorbance at 220 nm and electrochemical detection $E_1 = -1.0$ V and $E_2 = +0.2$ V. The acetonitrile gradient used is indicated by the dashed line.

graphed under the same conditions, and the six relevant peaks were manually collected. Those that were electrochemically negative were analyzed by amino acid analysis (peaks 1, 2 and 6) as shown in Table I. As can be seen, peptides present in peaks 1 and 2 are related to the region 10–12, 25–26 and the C-terminal Tyr residue of the ANF molecule; on the other hand, the peptide present in peak 6 is in all likehood released by trypsin and possesses an amino acid composition relating it to position 13–24. Its failure to be detected in a fashion similar to peaks 3, 4 and 5, by the electrochemical detector could very well be explained by oxidation of the disulfide bridge (with concomittant splitting) during or prior to the digest.

Finally, the three electrochemically positive peaks denoted 3, 4 and 5 were chemically reduced and alkylated using iodoacetic acid according to the scheme depicted in Fig. 1. Each peptide fraction was rerun under the same condition depicted in Fig. 3, except for the inclusion of a 5-min isocratic step to accommodate the reagents used during the chemical reaction, as seen in Fig. 4 by the presence of the two large peaks eluting at *ca.* 2 and 7 min. The UV chromatogram obtained by analyzing directly the reduced and alkylated peptides present in peaks 3, 4 and 5 are shown in Fig. 4, and the amino acid compositions of indicated peptides are listed in Table I. The amino acid composition data indicated that the ANF molecule was cleaved post Arg residues at positions 2, 9, 12 and 25 but also post Phe residues at positions 6 and 24; these cleavages suggest that the DCC-treated trypsin had a con-

TABLE I

AMINO ACID COMPOSITIONS OF PEPTIDES OBTAINED FROM TRYPSIN DIGESTION OF SYNTHETIC RAT ANF

Peaks are numbered according to their position in the chromatograms shown in Figs. 3 and 4. All values were obtained from 24-h hydrolysis in 5.7 *M* hydrochloric acid, 0.01% mercaptoethanol and a trace of phenol. The compositions were adjusted according to the underlined residues. CM-Cys corresponds to carboxymethyl-cysteine.

Amino acid	*Peak 1*	*Peak 2*	*Peak 3*				*Peak 4*				*Peak 5*	*Peak 6*
			A	*B*	*C*	*D*	*A*	*B*	*C*	*D*	*A*	
Asx	0.96	–	–	1.03	1.98	0.6	1.10	1.01	0.99	0.98	1.03	1.02
Ser	–	–	1.74	1.80	3.60	1.83	1.90	1.78	1.74	2.45	1.77	1.86
Glx	–	–	–	1.16	1.32	1.62	1.16	1.15	1.01	1.20	1.01	1.04
Gly	–	–	–	2.89	5.00	3.00	3.20	2.88	2.77	3.67	2.92	2.81
Ala	–	–	–	1.00	1.20	1.00	1.20	1.00	0.89	1.00	0.87	1.00
Ile	1.00	–	–	0.93	1.73	0.50	0.96	0.98	0.87	–	1.00	0.89
Leu	–	–	–	1.00	1.46	1.08	1.16	1.00	1.00	1.13	1.18	0.97
Tyr	0.4	1.00	–	–	1.01	0.50	–	–	0.71	0.85	1.05	–
Phe	–	–	1.00	0.93	1.79	0.76	1.00	0.94	0.60	0.49	0.93	0.96
Arg	1.04	1.12	–	0.94	3.90	–	0.91	–	0.71	–	0.82	–
CM-Cys	–	–	0.78	0.74	2.29	–	0.73	0.75	0.60	–	0.72	–
Localization	10–12 + 26	25–26	3–6	13–25	2–26	–	13–25	13–24	13–26	–	13–26	13–24
Yield (nmol)	13.6 + 5.01	5.4	4.5	1.9	0.43	0.24	0.58	2.77	0.37	0.10	0.45	1.19

taminating chymotryptic activity. In agreement with the scheme proposed (Fig. 1), one can see that peak 3, upon reduction and alkylation, yielded two peptides, denoted 3A and 3B corresponding to positions 3–6 and 13–25, respectively; obviously these had to be linked together in the native molecule in order to be released after cleavage of the bridge. Moreover, the presence of a peptide (peak 3C) coeluting in the first separation confirms the utility of the second HPLC run in order to clean the sought-after peptides. Peak 3D, because of its high UV absorbance and low peptide yield (240 pmol) is thought to be a contaminating peak arising from the chemical synthesis. Similar results were also obtained on chromatography and analysis of electrochemically positive peaks 4 and 5. Indeed, the presence of the tetrapeptide Ser-Ser-Cys-Phe, eluting at *ca.* 21.5 min, is seen to follow the presence of C-terminal peptides containing the Cys-21, as in the case of peptides 4A (13–25), 4B (13–24), 4C (13–26) and 5A (13–26). It thus seems that in these three peaks before reduction and alkylation, the major peptides are the tetrapeptide linked to peptide 13–25 (peak 3B), 13–24 (peak 4B) and 13–26 (peak 5A). Whereas the small amount of peptide 13–26 present in fraction 4 (peak 4C) is probably due to contamination by the closely eluting fraction 5, the most likely explanation for the presence of peptide 13–25 (peak 4A), previously found in the well-separated fraction 3, would be that, before reduction and alkylation, it was linked to the peptide 3–9 instead of the peptide 3–6. The peptide 3–9 is likely to be present in the other peaks, which were not analysed, during the purification of the reduced and alkylated fraction 4. It is also noteworthy that all

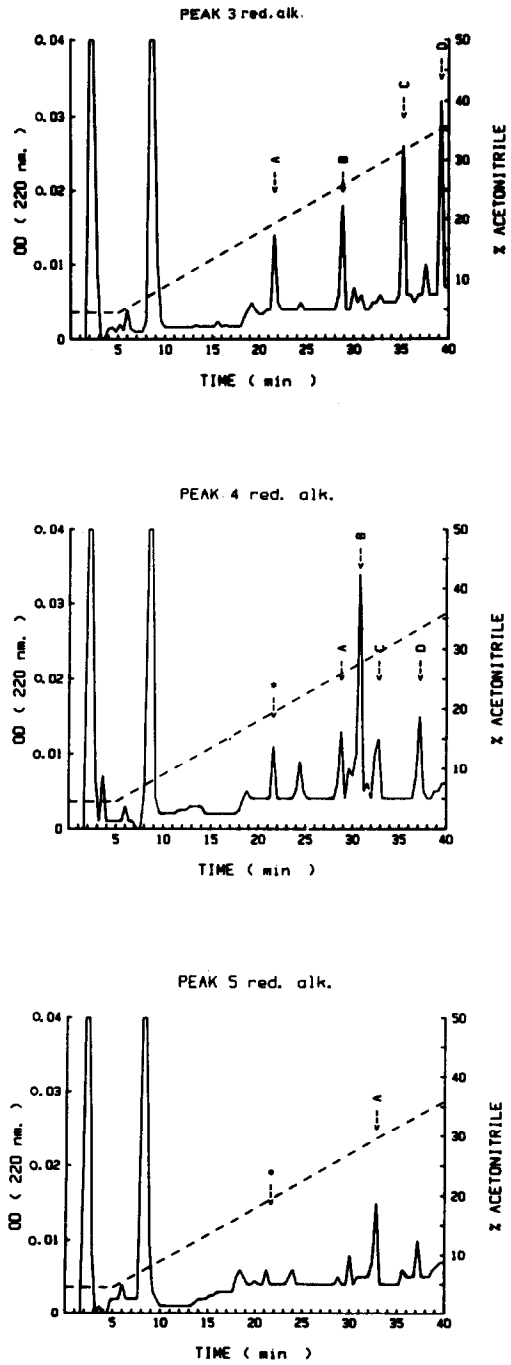


Fig. 4. Reversed-phase HPLC of the electrochemically positive peaks shown in Fig. 3 (peaks 3, 4 and 5) following chemical reduction and alkylation. The acetonitrile gradient used is indicated by the dashed line, and the amino acid compositions of the indicated peptides are listed in Table I.

peaks detected by the electrochemical detector indeed contained peptides that could subsequently be split on reduction and alkylation, thus warranting the validity of the proposed scheme.

CONCLUSIONS

This report demonstrates the validity of a new technique based on the specific detection of disulfide-containing peptides with an electrochemical detector. Indeed, in all cases of electrochemically positive peaks, it can be seen that, on reduction and alkylation, a single peak was split into various fragments containing the two half-cystine residues involved in the bridge. Even though it remains to be seen whether this approach could potentially be applied to proteins containing numerous disulfide bridges, results presented herein seem to favor such an approach. Moreover, preliminary data obtained through analysis of the pepsin digest of an Arg-specific protease are encouraging.

Furthermore, the presented results also point to the possibility of using the electrochemical detector in combination with UV absorbance as a dual-detector method to monitor the purification of disulfide bridge-containing peptides and also, to detect the presence, as seen in the case of peak 6, of oxidized material in either natural or synthetic mixtures. Considering that the application of ED to the peptide and protein field is only beginning, further work is clearly needed and the technique may, in time, represent a powerful tool in this field.

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