Journal of Chromatography, 443 (1988) 343–354
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1357

RAPID PEPTIDE MAPPING BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Short columns, packed with pellicular sorbents made of 2-μm fluid-impervious silica microspheres, were used at elevated column temperatures for rapid peptide mapping by high-performance liquid chromatography (HPLC). Enzymic digests of various proteins were chromatographed by gradient elution. In many cases the time of analysis was 10 min or less. In order to increase the retention particularly, that of short, polar peptides under such conditions, 1 m*M* octyl sodium sulfate or 5 m*M* hexyl sodium sulfate were added to the starting eluent. The length of the 4.6 mm I.D. columns was 30 or 75 mm, the sample load was in the range of 10–1000 pmoles. Highest analytical sensitivity was obtained at a flow-rate of 0.5 ml/min and room temperature, whereas for rapid analysis flow-rates of up to 2 ml/min were used at 80°C. This temperature allowed the use of relatively high flow velocities of the mobile phase without significant loss in efficiency. The method was highly reproducible, as shown by the results obtained by automated analysis of cyanogen bromide fragments of lysozyme at high speed. The quality of the rapid peptide maps compares favorably with that of maps obtained by standard reversed-phase HPLC methods, which require much longer analysis times.

INTRODUCTION

Peptide mapping is a widely used analytical technique for identification, characterization, and structural investigation of proteins and for isolation of the peptide components in the enzymic digests. It is essential for the determination of the amino acid sequence. The classical methods of peptide separation by thin-layer chromatography (TLC)[1] or electrophoresis[2] have largely been replaced by reversed-phase high-performance liquid chromatography (HPLC)[3–7], which yields increased sensitivity and higher resolution. Conventional HPLC techniques employ reversed-phase columns, packed with 5–10-μm alkyl-silica particles, and gradient elution for peptide mapping[8–11]; a chromatographic run for a peptide map often takes 2 h or more. Typically, nanomol quantities of a protein digest are chromatographed, although recently the sensitivity of the technique has been extended to the low pmol range[12–14].

The use of peptide mapping has gained prominence in applications such as the

0021-9673/88/$03.50

study of microheterogeneity[15], mutational variants[16–19], and isomers[20,21] present in purified proteins as well as the identification of site-specific glycosylation[22]. Peptide mapping by HPLC has also become a routine procedure in the determination of protein purity and is used for the quality control of r-DNA-derived proteins in biotechnology[15,23]. Consequently, there is great interest in reducing the analysis time required for peptide mapping by HPLC. High-speed chromatographic techniques that can separate and detect peptides at the pmol level would benefit structural studies on proteins at large.

The main goal of this work was to develop HPLC techniques for rapid peptide mapping by the approach we described recently for fast analysis of proteins and peptides[24]. An important feature of this technique is the use of short columns packed with micropellicular hydrocarbonaceous stationary phases, prepared from fluid-impervious monodisperse silica microspheres having a particle diameter of 2 μm. Such columns allow separation to be carried out at relatively high flow velocities and at elevated temperature, owing to the favorable mass transfer characteristics and stability of the pellicular sorbents[25–27]. As illustrated by the peptide maps of several proteins, the separation time can be reduced by a factor of 10 compared to conventional techniques without loss of efficiency and sensitivity.

EXPERIMENTAL

Materials

Cytochrome *c* (horse heart), lysozyme (chicken egg white), β-lactoglobulins A and B (bovine milk), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, N_α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated chymotrypsin, cyanogen bromide, dithiothreitol (DTT), and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, U.S.A.). r-Human growth hormone (r-hGH) and r-tissue plasminogen activator (r-tPA) were from Genentech (South San Francisco, CA, U.S.A.). Iodoacetic acid, formic acid, hexyl sodium sulfate and octyl sodium sulfate were obtained from Eastman Kodak (Rochester, NY, U.S.A.). HPLC-grade acetonitrile, reagent-grade orthophosphoric acid, and buffer salts were from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Instruments

A Series 400 pump from Perkin-Elmer (Norwalk, CT, U.S.A.) was used to pump the eluent through a heat exchanger coil, a Rheodyne Model 7025 injection valve with a 20-μl loop and the column; all of them were kept in a Model DL-8 constant temperature bath (Haake-Buchler, Saddlebrook, NJ, U.S.A.). The dead-volume of fittings and tubings was kept at a minimum and the flow-cell of the detector was pressurized. Most chromatograms were obtained by monitoring the column effluent at 210 nm and at 0.2 a.u.f.s. sensitivity with a Model LC-95 variable-wavelength detector (Perkin-Elmer) and were processed with a Model C-R3A integrator (Shimadzu, Columbia, MD, U.S.A.). The actual gradient profiles were determined with the column in place, and acetonitrile–water (95:5, v/v) as the starting eluent, and the same solution containing L-tryptophan (10 μM) as the gradient former. A tracing of the gradient was obtained at 210 nm. Automated analysis was carried out with the above system by adding a Model ISS-100 autosampler control unit and a Model LCI-100 integrator from Perkin-Elmer.

Columns

C_8 and C_{18} micropellicular sorbents (d_p = 2 μm), described earlier[24] were packed into 30 × 4.6 mm I.D. and 75 × 4.6 mm I.D. columns using a methanol slurry at 70 MPa. Propyl-silica (7 μm, 300 Å) from DuPont (Wilmington, DE, U.S.A.) was packed into a 50 × 7 mm I.D. column in the same way. All plumbing, fittings, and 0.5-μm frits were made of No. 316 stainless steel.

Chromatographic conditions

Sample injections were made to coincide with the commencement of the gradient, as established by tracer experiments described above. After the peptide separation was completed, the precolumn dead-volume was flushed out with the help of a purge valve, and the columns were regenerated by perfusion with the starting eluent, containing 1 m*M* octyl sodium sulfate, at a flow-rate of 3 ml/min. The regeneration time did not exceed 1.5 min. In the absence of instrumental constraint, it is likely to be shorter, due to rapid mass transfer characteristics of the micropellicular sorbents. The reversed-phase columns used in this study have not shown any sign of deterioration from use at 80°C with eluents containing ion pairing agents over a period in excess of 1500 h, as judged from the chromatograms of cyanogen bromide fragments of lysozyme run at regular time intervals.

Sample preparation

Reduction and S-carboxymethylation of proteins was carried out according to the procedure of Crestfield *et al.*[28], with the exception that β-mercaptoethanol was replaced by DTT. In typical experiments, the protein (5 mg) was denatured in a solution containing 8 *M* urea (2 ml) and reduced in the presence of DTT (10 m*M*) by incubation at 37°C for 2 h. Subsequently, the protein was alkylated by addition of iodoacetic acid (20 m*M*). After 10 min, the reaction mixture was dialyzed against 50 m*M* ammonium bicarbonate or subjected to chromatodialysis, as described below. Native or reduced and S-carboxymethylated proteins were digested with TPCK-treated trypsin or TLCK-treated chymotrypsin according to standard procedures[29]. The proteins (2 mg/ml) and trypsin or chymotrypsin at a substrate to enzyme ratio of 100:1, in a solution containing 100 m*M* sodium acetate, 10 m*M* Tris and 0.1 m*M* calcium chloride (pH 8.3), were incubated at 37°C. After 4 h, the reaction was arrested by adding 10% (v/v) orthophosphoric acid. The digests were stored at 4°C. Cyanogen bromide cleavage of lysozyme was carried out by the procedure of Chen-Kiang *et al.*[30]. Amounts of 5 mg of the alkylated lysozyme and 200 mg of cyanogen bromide were added to 2 ml of 70% (v/v) formic acid, and the mixture was incubated in the dark at 22°C for 24 h.

RESULTS AND DISCUSSION

Introduction of pellicular sorbents and the use of elevated column temperature played an important role in demonstrating the feasibility of increasing speed, efficiency, and sensitivity of HPLC[24–27]. Short columns, packed with micropellicular sorbents[24,31–37], increase the speed of chromatographic analyses further. In micropellicular stationary phases the retentive material forms a thin layer on the surface of fluid-impervious microspheres. In comparison to conventional porous sorbent par-

ticles of the same diameter, the pellicular configuration, allows faster equilibration of the eluite between the two phases and imparts higher stability to the column. Consequently, high flow velocities without loss of efficiency can be used with concomitant increase in the speed of analysis. The advantages of operating the column at elevated temperature have also been demonstrated recently[24,38]. The stability of the column packed with micropellicular sorbents permits the use of elevated temperature, without loss in column efficiency. As the viscosity of the mobile phase decreases with increasing temperature, relatively high flow velocities can be used even with columns containing 2-μm particles, without exceeding the pressure limitations of standard HPLC equipment.

Modification and degradation of proteins

In the preparation of enzymic digests the protein is first denatured, the disulfide bridges are reduced and the sulfhydryl groups are allowed to react with iodoacetic acid. In order to follow the time-course of protein degradation or modification, we used rapid HPLC analysis. The three chromatograms in Fig. 1 are "snapshots", illustrating the composition of the tryptic digest of cytochrome *c* at different times. The results suggest that the rapid analytical technique discussed here can also be useful in monitoring the time course of chemical reactions, and the high analytical speed allows information to be obtained almost instantaneously. This may be important, for instance, when the reaction must be stopped or another reagent must be added at a certain stage of the reaction.

Over the past few years we have used reversed-phase columns and HPLC instrumentation for the isolation of modified proteins from reaction mixtures and termed the technique "chromatodialysis". usually 1–2 ml of the reaction mixture, containing 3–5 mg of protein, are loaded onto an alkyl-silica column (100 × 4.6 mm I.D.) by using a sample loop of appropriate volume. The column is perfused with water to remove salt and other low-molecular-weight components except the proteinaceous ones, which are eluted in a subsequent step with acetonitrile containing 5% (v/v) water and 0.1% (v/v) TFA. The chromatogram in Fig. 2 illustrates the process for the recovery of cyanogen bromide fragments of lysozyme. Similar procedures for desalting of proteins[39] and nucleotides[40] have been published recently.

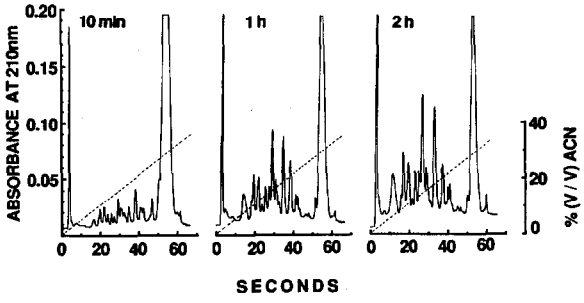

Fig. 1. Chromatograms illustrating the time course of cytochrome *c* degradation by trypsin. Column, micropellicular C_8 silica, 30 × 4.6 mm I.D.; eluent A, 0.1% (v/v) TFA in water; eluent B, 5% water (v/v) and 0.1% (v/v) TFA in acetonitrile (ACN); flow-rate, 5 ml/min; temperature, 80°C; initial column inlet pressure, 31 MPa. Aliquots (10 μl) taken from the reaction mixture at the incubation time indicated, were mixed with 10 μl of eluent A and injected directly by using a 20-μl loop.

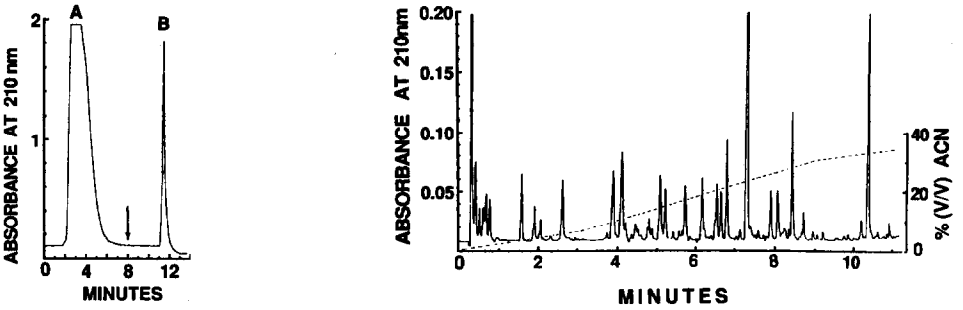

Fig. 2. Chromatodialysis of the cyanogen bromide fragments of lysozyme. Column, 7-μm propyl-silica, 50 × 7 mm I.D.; flow-rate, 2 ml/min; temperature, 25°C; sample, 2 ml of reaction mixture, containing 5 mg of reduced and S-carboxymethylated lysozyme. The sample was introduced into the column, equilibrated with water, and the flow of water was continued until formic acid and salt (peak A) were washed off. Then the mobile phase was changed to 95% (v/v) acetonitrile and 0.1% (v/v) TFA, (arrow), and the protein components (peak B) were recovered and lyophylized.

Fig. 3. Tryptic map of r-hGH. Column, micropellicular C_{18} silica, 75 × 4.6 mm I.D.; eluent A, 5 m*M* hexyl sodium sulfate and 50 m*M* sodium dihydrogenphosphate in water, pH adjusted to 2.8 with orthophosphoric acid; eluent B, 50 m*M* of sodium dihydrogen phosphate in 60% (v/v) acetonitrile (ACN), pH adjusted to 2.8 with orthophosphoric acid; flow-rate, 2 ml/min; temperature, 80°C; sample, 4 μg of r-hGH digest in 20 μl.

Peptide maps

Peptide maps obtained by rapid HPLC analysis of completely digested proteins are depicted in Figs. 3–6. The chromatograms in Figs. 3 and 4 show the separation of the peptides in tryptic digests of r-hGH and β-lactoglobulin A with satisfactory resolution in about 12 min. The peptide map of the chymotryptic digest of β-lactoglobulin A is shown in Fig. 5. Comparison of Figs. 4 and 5 confirms that chymotrypsin digestion generates more fragments than trypsin digestion. The chromatogram of the tryptic digest of r-tPA (Fig. 6) shows the greatest number of components

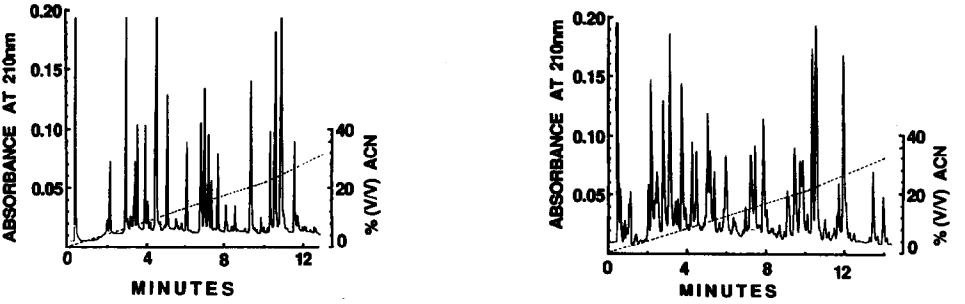

Fig. 4. Tryptic map of β-lactoglobulin A. Column, micropellicular C_{18} silica, 75 × 4.6 mm I.D.; flow-rate, 1.8 ml/min; sample, 3.5 μg of reduced and S-carboxymethylated β-lactoglobulin A digest in 20 μl. Eluents as in Fig. 3, except 1 m*M* octyl sodium sulfate was present in eluent A.

Fig. 5. Chymotryptic map of β-lactoglobulin A. Sample, 3.5 μg of reduced and S-carboxymethylated β-lactoglobulin A digest in 20 μl. Experimental conditions were as in Fig. 4, except the protein was digested with TLCK-treated chymotrypsin.

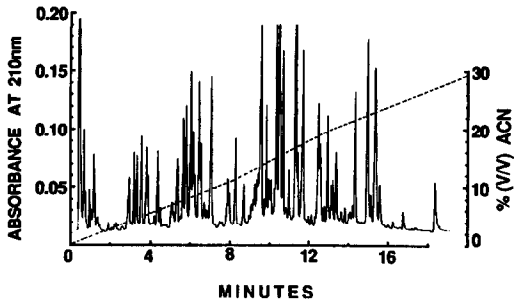

Fig. 6. Chromatographic separation of tryptic peptides of r-tPA. Experimental conditions as in Fig. 4. The protein was digested with TPCK-treated trypsin. Sample, 5 μg of reduced and S-carboxymethylated r-tPA digest in 20 μl.

in comparison to the other peptide maps presented here because of the relatively high molecular weight of this protein [66 kilodalton (kD)]. Nevertheless, the number of peaks (52) that were separated in less than 20 min and the quality of the peptide map is comparable to that obtained by conventional techniques which take much more time[8–14,20–22]. It is noted that the favorable mass transfer associated with the micropellicular configuration facilitates rapid reequilibration of the column after a gradient elution. Thus, time is gained by both faster separation and faster column regeneration.

Operational variables

The micropellicular reversed-phase columns used in this study could be used satisfactorily for peptide analysis in a wide range of operating conditions. The stability of this kind of stationary phase allowed LC to be performed at elevated temperature without untoward effects, such as decrease in column permeability or loss in efficiency. Due to the small particle size (2 μm) of the packing material, the inlet pressure of a 30 × 4.6 mm I.D. column was 20 MPa with water at a flow-rate of 1 ml/min at 22°C. We have shown previously[24] that the viscosity of water–acetonitrile mixtures decreases 3-fold upon increasing the temperature from 20 to 80°C. Thus, by such an increase in column temperature, the flow velocity of the mobile phase can be trebled without increasing the inlet pressure of the column. Since the micropellicular column packings can, by virtue of favorable mass transfer properties, be used at high flow velocities without loss in resolution, it was advantageous to use short columns at elevated temperature for high speed analysis. The benefits of micropellicular packings manifest themselves in faster separations and better recoveries than those obtained by using columns packed with conventional porous sorbents[24,31–37], even at room temperature. High analytical sensitivity is observed at low flow velocities when there is no obvious advantage to operate the column at elevated temperature. The loss of sensitivity at high flow velocities can usually be compensated for by increasing the size of the sample. In gradient elution, the shape of the gradient, the gradient time and volume, have the most significant effect on the separation. The results of our investigations with the micropellicular reversed-phase columns are in agreement with the theoretical predictions and the findings of others[34–36,41]. The only additional variable in our case was the column temperature. We found that by op-

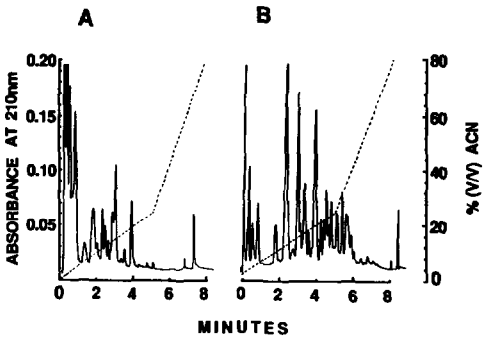

Fig. 7. Peptide maps of lysozyme without (A) and with (B) ion-pairing agent in the eluent. Column, micropellicular C_8 silica, 30 × 4.6 mm I.D.; eluent A, 0.1% (v/v) TFA in water; eluent B, 95% (v/v) acetonitrile and 0.1% TFA in water. The eluent used for chromatogram B contained 5 m*M* hexyl sodium sulfate, flow-rate, 3 ml/min; temperature, 80°C; sample, chymotryptic digest of 5 μg of reduced and S-carboxymethylated lysozyme in 20 μl.

timizing the above-mentioned variables in each case, the analysis time with a given chromatographic system could be consistently reduced by increasing column temperature. The retention in most modes of LC separations decreases with increase in temperature and it has been shown that the retention enthalpies for proteins are several fold greater than those observed for small molecules in reversed-phase chromatography[24]. Thus, during analysis of protein digests at high temperature, some of the sample components particularly, the short, polar peptides may not exhibit satisfactory retention. Fig. 7A shows such an example where a number of components in tryptic digest of lysozyme were eluted in the void fraction when the separation was

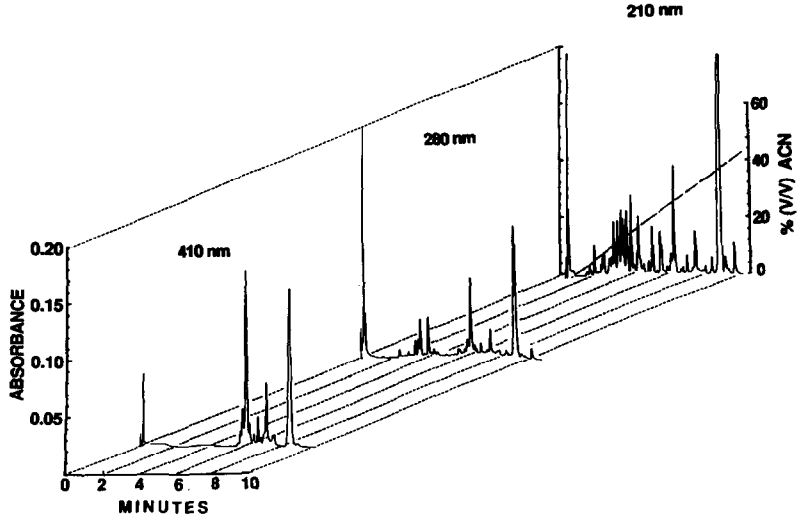

Fig. 8. Effect of detection wavelength on the chromatograms of chymotryptic peptides of cytochrome *c*. Conditions as in Fig. 1 except the flow-rate was 3 ml/min and the column effluent was monitored at 210, 280 and 410 nm. Sample, 5 μg of cytochrome *c* digest in 20 μl.

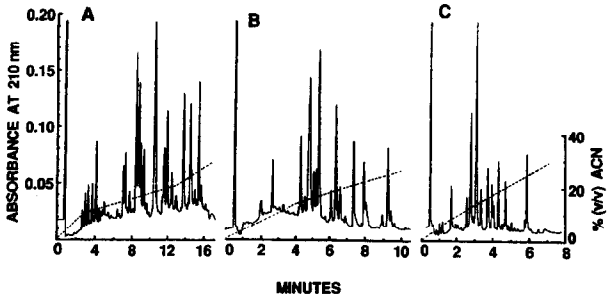

Fig. 9. Effect of temperature and flow-rate on the separation of tryptic fragments of β-lactoglobulin B. Conditions as in Fig. 4, but chromatograms A, B and C were obtained at 25, 50 and 80°C and at flow-rates of 0.8, 1.3 and 2.0 ml/min, respectively. The initial column inlet pressure was 34.3 MPa in each case. Sample, 4 μg of reduced and S-carboxymethylated β-lactoglobulin B digest in 20 μl.

carried out at 80°C. Addition of small amounts of a haeteron, such as hexyl sodium sulfate to the mobile phase caused significant increase in the retention by ion-pairing effect (Fig. 7B). Monitoring of column effluent at different wavelengths provides useful information for spectral identification of various peptides in the digests of protein samples. As seen in Fig. 8 those peptides in the tryptic digest of cytochrome *c* which contain heme, or the aromatic amino acids such as tryptophan and tyrosine are selectively detected at 410 and 280 nm, respectively whereas monitoring at 210 nm allows detection of all peptide components in the sample. The chromatograms in Fig. 9 illustrate the effect of column temperature on the separation of the tryptic digests of β-lactoglobulin B at a fixed column inlet pressure. The saving in analysis time with elevated temperature is most conspicuous and is a consequence of the increased flow-rate. Changes in the elution pattern are noted, because the gradient shape and gradient time were varied to obtain maximum separation of the sample components under different conditions. Since the sample size was the same in each case, the sensitivity is higher at 25°C due to the lowest flow velocity. The results demonstrate that the column temperature can be a significant operational variable in LC. However, the construction of the instrument, did not allow us to take full advantage of the higher speed obtained at higher temperature. Novel instrumental design is required to effect rapid mixing and heating of the mobile phase in a system with a very low dead-volume. Accordingly, the time constants of other elements of the liquid chromatograph should also be reduced. As HPLC is widely used in routine analytical work, novel approaches that allow rapid separations can help to overcome one shortcoming of column chromatography *viz.*, that samples must be processed sequentially, and simultaneous analysis of multiple samples as in TLC or electrophoresis is impossible. However, by sufficiently reducing the analysis time, HPLC could also be quite competitive in analytical productivity.

Sensitivity

Most chromatograms shown here were obtained with digest samples corresponding to 5 μg of protein (about 100 pmol of a 50-kD protein), which represents the lower sensitivity limit in peptide mapping by conventional HPLC. The effect of sample size is illustrated in Fig. 10 by the tryptic maps of β-lactoglobulin B, obtained

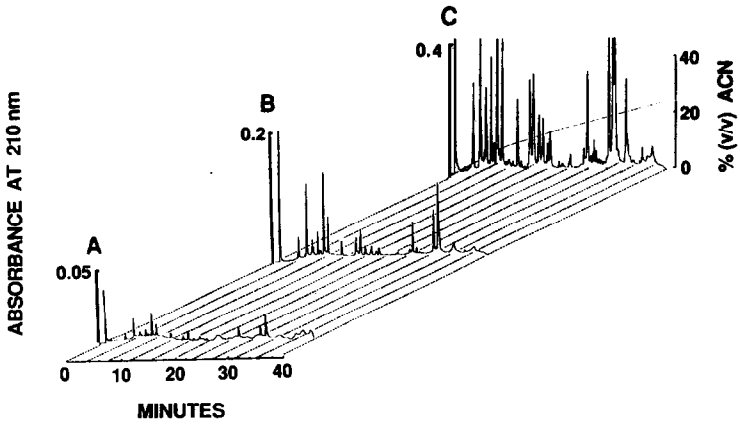

Fig. 10. Effect of sample size on the tryptic maps of β-lactoglobulin B. Conditions as in Fig. 4 except the flow-rate was 0.5 ml/min. Sample: A, 10 pmol; B, 162 pmol; and C, 972 pmol of reduced and S-carboxymethylated β-lactoglobulin B digest in 20 μl.

by using a shallow gradient and high temperature. An example of a high-sensitivity peptide map is shown in Fig. 10A. The chromatogram was obtained with 10 pmol of protein digest at a detector sensitivity of 0.05 a.u.f.s. Even with such a small sample, the chromatogram reveals all major peptide components, and the observed sensitivity is about five times better than that reported for peptide maps by conventional HPLC techniques[12]. Since amino acid analysis can be performed routinely at picomol level[42–45], peptide chromatography at comparable sensitivity is required to exploit fully the potential of microsequencing technology, particularly for proteins that are available in only minute amounts. It is expected that by the use of micropellicular sorbents and columns of 0.5–1.0 mm I.D., reproducible peptide maps could routinely be obtained with 1 pmol of protein digest.

Fig. 10B shows a chromatogram obtained with 162 pmol of digest, (a typical

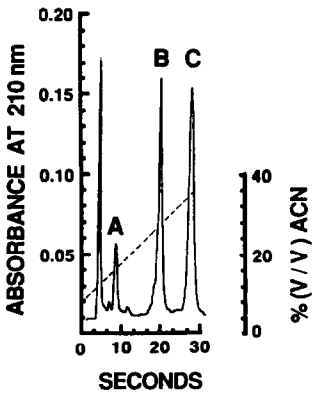

Fig. 11. Chromatograms of the cyanogen bromide fragments of lysozyme. Conditions as in Fig. 1 except the flow-rate was 4 ml/min. Sample, 3 μg of the reduced and S-carboxymethylated lysozyme digest in 20 μl.

TABLE I

REPRODUCIBILITY OF THE DATA OBTAINED WITH THE AUTOMATED SYSTEM UNDER CONDITIONS GIVEN IN FIG. 11

The number of injections was 12.

	Peak	*Mean*	*S.D.*	*R.S.D. (%)*
Retention time (s)	A	8.3	0.05	0.6
	B	16.7	0.06	0.4
	C	27.2	0.03	0.1
Peak area	A	3.32	0.09	2.7
(integration units $\times 10^{-5}$)	B	15.94	0.30	1.9
	C	24.97	0.71	2.8

sample size in peptide mapping by HPLC). Upon increasing the sample to 972 pmol no loss in resolution was observed (*cf.* Fig. 10B and C). The chromatogram obtained with a large sample size (*cf.* Fig. 10C) reveals the presence of several minor components which are not detected under the standard conditions used in peptide mapping. As the resolution was not decreased by increasing the sample, this approach can be used for the analysis of protein contaminants present in trace amounts in highly purified proteins.

Reproducibility

Treatment of reduced, S-carboxymethylated lysozyme with cyanogen bromide yields three peptides, which can be separated in 30 s, as shown in Fig. 11. In order to investigate the precision of the analysis at such a high speed, the chromatographic system was automated by incorporating an autosampler, which controlled the gradient formation, sample injection, and integrator. The sample was injected twelve times, and the retention times and peak areas were analyzed for statistical significance according to Godel *et al.*[46]. The results in Table I show that the average coefficients of variation for retention time and peak area were better than 0.4 and 2.5%, respectively. This level of precision obtained with an *ad hoc* experimental set up without optimization, is considered satisfactory for many peptide analyses.

CONCLUSION

The use of short columns packed with non-polar micropellicular sorbents as well as relatively high flow velocities and elevated temperature resulted in significant reduction in the analysis time for peptide mapping. Rapid mass transfer resulting from the pellicular configuration and elevated temperature is mainly responsible for the rapid separation and column reequilibration that characterize such systems. Moreover, the significance of rapid HPLC analysis goes far beyond this application and is expected to have great potential in obtaining quick analytical information, *e.g.* in the monitoring of process streams. The method is suitable for routine analysis at high sensitivity as well as with large sample load for the detection of trace impurities in purified proteins. Appropriate changes in the instrumental design are necessary to take full advantage of this approach.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. W. Hancock of Genentech for invaluable discussions and for generous gifts of r-hGH and r-tPA. The assistance of Dr. M. Dong of Perkin-Elmer in setting up the equipment for automated peptide analysis is gratefully acknowledged. This work was supported by Grants Nos. CA 21948 and GM 20993 from the National Institutes of Health, U.S. Department of Health and Human Services.

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