Journal of Chromatography, 317 (1984) 41–54
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 472

LIGAND-EXCHANGE CHROMATOGRAPHY ON COPPER(II)-MODIFIED SILICA GEL

IMPROVEMENTS AND USE FOR SCREENING OF PROTEIN HYDROLYZATE AND QUANTITATION OF DIPEPTIDES AND AMINO ACID FRACTIONS

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SUMMARY

Improvements in the copper(II) modification of silica gel, the stability of the analytical column by use of a guard column, the convenient UV detection at 254 nm of amino acids and peptides as their copper(II) complexes and the use of gradients make ligand-exchange chromatography a simple method for qualitative screening of protein hydrolyzates and quantitation of free amino acids and dipeptides in a complex polypeptide mixture. Examples are given of the chromatography of polyenzymatic ovalbumin hydrolyzates.

INTRODUCTION

Ligand-exchange chromatography on copper(II)-modified silica gel has been used for isocratic separations of amino acids[1] and small peptides[2]; the degradation of Met-enkephalin by peptidases present in striatum extracts has thus been followed by direct injection of aliquots of incubation medium[3].

Two major improvements have been achieved by the use of copper(II)-modified silica gel instead of conventional organic polymers. Fig. 1 is an example of the separation of common acidic and neutral amino acids on a copper(II)-modified silica gel micro-column[4], showing the high efficiency and selectivity obtained by combining ligand-exchange chromatography and micro-column technology; the technique can easily be adapted to a specific problem by varying the two main parameters, *i.e.*, the water content and ammonia concentration in the mobile phase, as described[1].

However, some drawbacks remain for routine applications, such as the slow dissolution of the silica backbone in basic and water-rich mobile phases, and the incompatibility between UV detection at 210 nm and gradient runs, because of the great difference between the absorbances of water and acetonitrile, even of the best grade.

We report here some improvements concerning the copper(II) modification of

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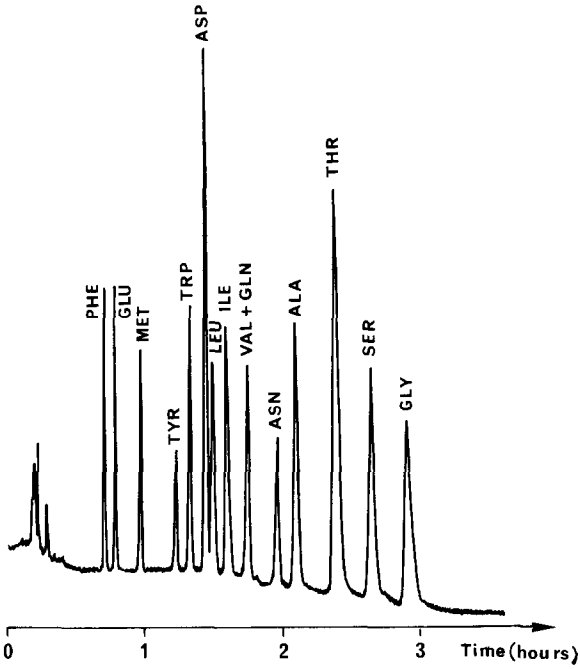

Fig. 1. Separation of neutral and acidic amino acids on a copper(II)-modified silica gel micro-column. Column: 100 × 0.1 cm I.D. Packing: copper(II)-modified Zorbax, particle diameter, d_p = 7 μm. Mobile phase: water–acetonitrile (50:50)–0.5 *M* ammonia. Temperature: 50°C. Pressure: 300 bar. Detection: UV at 210 nm. Sample: 1 μl.

pure silica gel, the stability of the analytical columns and the detection of copper(II)-complexed solutes. These improvements allowed us to devise gradient methods, which seem to be the first used in ligand-exchange chromatography (except in the step gradient mode), and to obtain chromatograms of protein hydrolyzates containing many peptides and amino acids, giving information on the quality of the hydrolysis.

We also devised a method of ligand-exchange chromatography on copper(II)-modified silica gel columns, eluted by mobile phases containing no acetonitrile, in order to obtain separations of polypeptide mixtures in families of free amino acids, dipeptides and other peptides previously observed by Rothenbuhler *et al.*[5] with the use of conventional ligand-exchange chromatography.

EXPERIMENTAL

Chromatographic apparatus

A Spectra-Physics Model 8000 was used for all our experiments (Spectra-Physics, Santa Clara, CA, U.S.A.), in conjunction with various UV detectors: Altex Model 153 (Beckman, Berkeley, CA, U.S.A.); Spectro Monitor III (LDC, Riviera Beach, FL, U.S.A.); Model SF769 (Kratos, Westwood, NJ, U.S.A.).

Chemicals and packing

Experiments were performed with LiChrosorb Si 60, mean particle size 7 or 10 μm (Merck, Darmstadt, F.R.G.), or Zorbax, mean particle size 7 μm (Du Pont, Wilmington, DE, U.S.A.). The guard columns were packed with Kieselgel 60, mean particle size 0.2–0.5 mm (Merck). These silica gels were modified with Cu(II) by percolation or by a batch technique, as described below.

Mobile phases were made up with distilled on "quartz" water (Quartex, Paris, France) and HPLC-grade acetonitrile (Prolabo, Paris, France). The eluting base was ammonia (R.P. Normapur; Prolabo) or N,N,N′,N′-tetramethylethylenediamine (TMED) (Merck). Copper(II) ions (1 ppm as copper sulphate) were added in mobile phases, regardless of composition, both as compensation for the slow leakage of Cu(II) from the column and as an aid to amino acid detection, as described below.

The amino acids were from Merck and the peptides from Serva (Heidelberg, F.R.G.). The polypeptide mixtures were from Laboratoire R. Bellon (Monts, France), obtained by the polyenzymatic degradation of ovalbumin; these mixtures consisted essentially of small peptides (di- to pentapeptides).

All other chemicals were from Merck or Prolabo.

Copper(II)-modified silica gel columns and guard columns

Stainless-steel columns of various lengths and 4.8 mm I.D. were packed by the slurry technique with LiChrosorb Si 60. After packing, the displacing solvent (ethanol) was removed from the slurry reservoir (20 ml), which was then filled with a $2 \cdot 10^{-1}$ *M* copper(II) sulphate–1 *M* ammonia solution. This solution was forced through the column at a moderate pressure (100–200 bar). The effluent was first ethanol (void volume), then pure water (reaction of silanol groups with ammonia), then 1 *M* ammonia solution free of Cu(II) [copper(II) equilibration of the column], then the incoming blue solution. The slurry reservoir was emptied again and filled with pure water or a dilute ammonia solution to wash the column, after which the column was equilibrated with the mobile phase used or kept under ethanol or acetonitrile.

The Kieselgel used for the guard column was modified with Cu(II) by a batch technique: an unweighed quantity of Kieselgel was treated with an excess of the $2 \cdot 10^{-1}$ *M* copper(II) sulphate–1 *M* ammonia solution (the modification is performed in a few seconds), then washed twice on a filter-paper with water or a dilute ammonia solution, then air-dried and kept in a bottle. Guard columns (usually 10 × 0.7 cm) were dry-packed and inserted between the chromatographic pump and the injector.

When TMED was used instead of ammonia, the silica gels used for the analytical and guard columns were treated exactly in the same way except that ammonia was replaced by TMED (at the same concentration). The resulting copper(II) contents of the equilibrated columns were 0.75 mmol per gram of LiChrosorb for the $Cu(NH_3)$ silica column and 0.55 mmol per gram of LiChrosorb for the Cu(TMED) silica column.

RESULTS AND DISCUSSION

Column stability and reproducibility

In previous work[1], a slow deterioration of the silica gel matrix was mentioned, and it is well known that silica gel is soluble in basic aqueous media. The solubility

of silica gel in ammonia–water–acetonitrile mixtures has been studied in our laboratory[6], and it was concluded that the deterioration was slow with these mixtures and that the solubility decreased rapidly with increasing acetonitrile percentage. A guard column (10 × 0.7 cm), filled with copper(II)-modified silica gel and inserted between the pump and the injector, allows sufficient presaturation of the mobile phase with silicate ions to prevent the analytical column from slowly dissolving.

Two sets of experiments have been carried out to check the stability and reproducibility. The results are shown in Figs. 2–4. For the first set, a 15 × 0.48 cm I.D. analytical column was percolated with a mobile phase of moderate strength, night and day for 1 week, and an injection of a text mixture of seven amino acids was made every 2 h. Fig. 2 shows the retention times of amino acids *versus* the time of the injection. Although a total volume of 24 l of mobile phase had been percolated through the column, no loss of efficiency was found (the column head remained

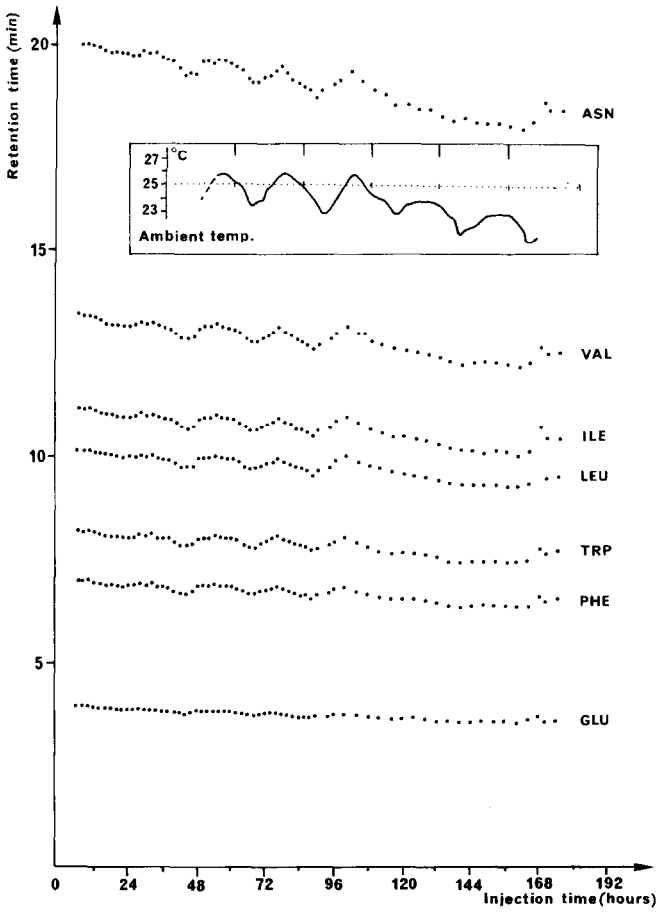

Fig. 2. Multiple injections of a test mixture of seven amino acids into a copper(II)-modified silica gel column. Column: 15 × 0.48 cm I.D. Packing: copper(II) modified LiChrosorb Si 60, d_p = 7 μm. Mobile phase: water–acetonitrile (50:50)–0.15 *M* ammonia, 1 ppm of Cu^{2+}. Flow-rate: 1.7 ml/min. Detection: UV at 254 nm. Sample: 30 μl containing a few μg of each amino acid.

intact), demonstrating the adequacy of the guard column. Small variations in retention times shown in Fig. 2 are closely correlated with the ambient temperature.

For the second set of experiments the 15 × 0.48 cm I.D. analytical column was percolated with a mobile phase of high strength, night and day for 2 weeks, and an injection of a test mixture of nine amino acids was made every 6 h. Fig. 3 shows the capacity factors, k', of amino acids *versus* the time of the injection. Fig. 4 shows three chromatograms of the test mixture, obtained after percolation of 3, 13 and 19 l of mobile phase through the column. Retention times slowly decreased and we observed a slow decrease in selectivity, but no decrease in efficiency. A slow decrease in the copper(II) content of the column is responsible for this phenomenon; when retentions or selectivities become unacceptable[7], it may be compensated by increasing the copper(II) concentration in the mobile phase, or by percolation with a small volume of the solution used for modification.

We conclude therefore that a guard column of crude particles of copper(II)-modified silica gel is suitable for stabilizing the analytical column and for the reproducibility of the results by ligand-exchange chromatography on copper(II)-modified silica gel. The addition of Cu^{2+} to the mobile phases causes no problems, as it compensates for leakage of Cu(II) and helps in amino acid detection, as shown below.

Detection of amino acids and peptides as their copper(II) complexes

Post-column derivatization with ninhydrin or other reagents is commonly used for the detection of amino acids and peptides. UV spectrophotometry of underivatized amino acids and peptides at a low wavelength (210 nm) has been used in re-

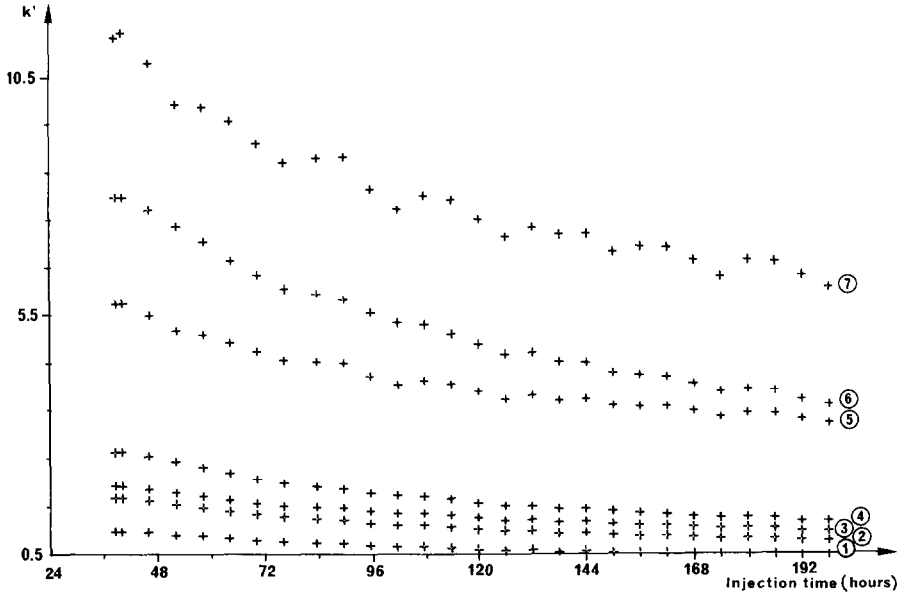

Fig. 3. Multiple injections of a test mixture of nine amino acids into a copper(II)-modified silica gel column. Mobile phase: water–acetonitrile (50:50)–1.4 *M* ammonia, 1 ppm of Cu^{2+}. Flow-rate: 2ml/min. Other details as in Fig. 2. Sample: 1 = Tyr (4.5 μg); 2 = Gln (2.4 μg); 3 = Val + Thr + Pro (12 μg); 4 = Ser (3 μg); 5 = Lys (6.6 μg); 6 = His (15 μg); 7 = Arg (30 μg). k' = Capacity factor.

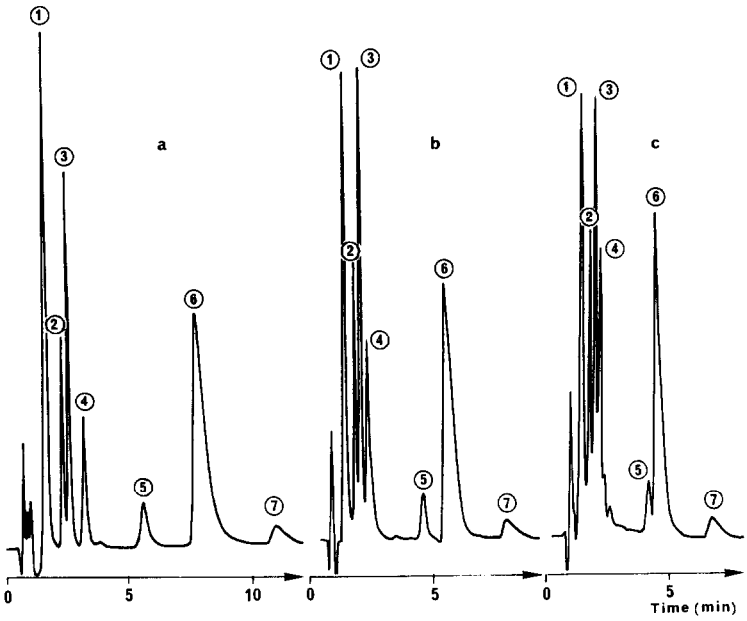

Fig. 4. Evolution of the chromatograms during the night-and-day elution of a copper(II)-modified silica gel column by a high-strength mobile phase. Conditions as in Fig. 3. a, After percolation of 3 l of mobile phase through the column; b, after percolation of 13 l; c, after percolation of 19 l.

versed-phase chromatography[8] and in ligand-exchange chromatography[1,2,9]. This low-wavelength detection has two drawbacks: it is of low selectivity, because numerous substances show absorption at 210 nm; it is very sensitive to temperature or solvent changes; the use of gradients (NH_3 or water) is impossible with conventional detectors.

The detection of copper(II) complexes of amino acids and peptides has been suggested by Walton[10], and was used in reversed-phase chromatography by Grushka and co-workers[11–13] and in ligand-exchange chromatography for enantiomeric separations by Davankov and co-workers[14,15]. We have also used this method and report here some results concerning the detectability of copper(II) amino acid complexes in ligand-exchange chromatography.

Fig. 5 shows the dependence of peak area upon detection wavelength for some amino acids, the copper(II) concentration being kept constant at $2 \cdot 10^{-5}$ *M*. The absorbance of two sugars is reported too, as they are known to form substitution-labile complexes with cuprammonium ions, making them visible in the UV region[16]. Absorptivities increase with decreasing wavelength down to 225 nm, and then decrease (shown for glucose, saccharose and aspartic acid). The decrease is due much more to the large variation mobile phase absorbance than to a decrease in absorbance of the complexes. A few ppm of Cu^{2+} in the mobile phases then facilitate the UV detection of amino acids and peptides at a wavelength between 230 and 260 nm. As the noise level generally decreases with increasing wavelength for most detectors, and as the absorption level of the mobile phase is approximately constant, this type of detection will be useful for routine analysis and for gradient elution.

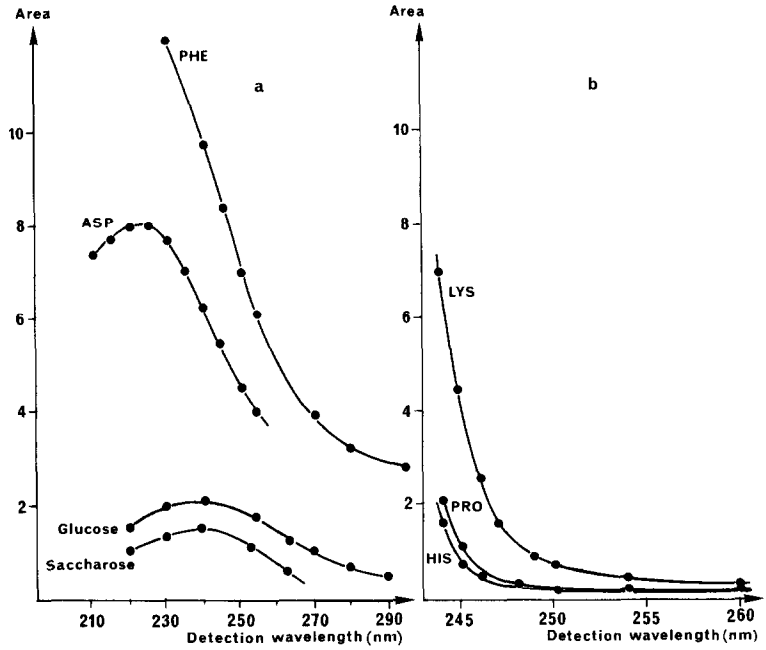

Fig. 5. Dependence of peak area upon detection wavelength for some amino acids and sugars. Mobile phase: water–acetonitrile: a, (52:48)–0.15 *M* ammonia; b, (50:50)–1 *M* ammonia; 1 ppm of Cu^{2+}. Flow-rate: 2 ml/min. Other details as in Fig. 2.

Fig. 6a shows a chromatogram obtained by injection of 80–500 pmol of amino acids, the detection being performed with a fixed-wavelength detector (254 nm). Fig. 6b shows a chromatogram obtained by injection of 50 pmol to 1.4 nmol of amino acids, detection being performed with a multiwavelength detector at 235 nm. Despite the decreasing absorptivity shown in Fig. 5, the detectability remains good at 254 nm, and it allows routine detection of amino acids with ordinary UV detectors. The linearity of the detector response is adequate when only a few ppm of Cu^{2+} are added to the mobile phases used. These copper(II) ions have an additional effect. They prevent the slow diminution of the copper(II) content of the stationary phase when used with mobile phases of high strength.

Use of gradients in ligand-exchange chromatography

In earlier work[1,6,17], we have studied the effect of the mobile-phase composition on the retention times of amino acids and of several peptides. It became apparent that, to give good results, the mobile-phase composition must depend on the size and on the hydrophilic and complexing properties of the solutes studied. Therefore, if a complex mixture of peptides and amino acids is to be resolved in the same chromatogram, it will be best to use a gradient, starting with a mobile phase poor in water and ammonia (elution of large, hydrophobic and weakly complexing peptides), then increasing these two parameters, and eluting solutes in an order related to their hydrophilic and complexing properties. The resulting chromatogram will depend on the time variations of the water percentage and of the ammonia concen-

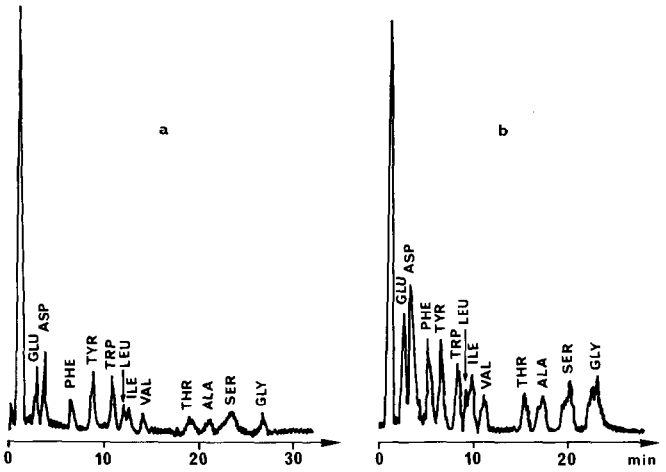

Fig. 6. Limit of detection of neutral and acidic amino acids as their copper(II) complexes in ligand-exchange chromatography. Mobile phase: water–acetonitrile (52:48)–0.15 *M* ammonia, 1 ppm of Cu^{2+}. Flow-rate: 2 ml/min. Detection: UV, 254 (a), 235 nm (b). Other conditions as in Fig. 2. Sample (nmol injected):

	Glu	Asp	Phe	Tyr	Trp	Leu	Ile	Val	Thr	Ala	Ser	Gly
a	0.08	0.09	0.22	0.12	0.17	0.27	0.27	0.3	0.3	0.4	0.3	0.47
b	0.08	0.05	0.14	0.05	0.02	0.36	0.36	0.51	0.25	0.82	1.2	1.4

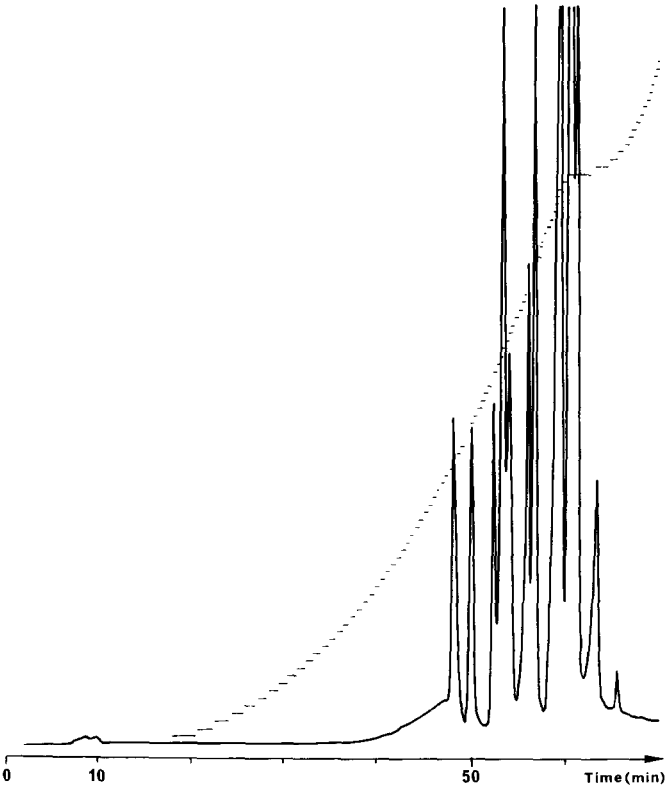

Fig. 7. Chromatogram of common amino acids. Mobile phase: A, water–acetonitrile (10:90)–0.1 *M* ammonia, 1 ppm of Cu^{2+}; B, water–acetonitrile (60:40)–0.95 *M* ammonia, 1 ppm of Cu^{2+}. Gradient as shown by the dotted line. Flow-rate: 2 ml/min. Other details as in Fig. 2. Sample: 30 μl containing 1000 μg of the test mixture.

tration in the mobile phase. A typical gradient[18] is presented here. It has a non-linear shape, varying from 10% water–0.1 *M* NH_3 in acetonitrile to 60% water–0.95 *M* NH_3 in acetonitrile in 70 min (1 ppm of Cu^{2+} was added for UV detection).

Fig. 7 shows the resulting chromatogram of common amino acids. They are not equally resolved, but are eluted in the last 20 min of the gradient, thus leaving the first 50 min for peptides elution.

Fig. 8 shows a composite chromatogram, obtained by multiple injections of pure or grouped solutes in order to characterize various time zones of the gradient. The 45–70 min zone contains all the amino acids, the very hydrophilic peptides, like Ser-Ser-Ser or Gly-Gly-Gly, and the peptides having basic amino acids in their sequence. The central zone (15–45 min) contains essentially the dipeptides. The 0–15 min zone contains hydrophobic peptides, like Phe-Phe or Ala-Ala-Ala.

We can comment on these results as follows: all tripeptides more hydrophobic than Ala-Ala-Ala, and all peptides of larger size, having no basic amino acids in their sequence and not consisting essentially of Ser, Thr, Gln or Asn, will be eluted before Ala-Ala-Ala. Dipeptides (except the very hydrophobic ones and basic ones), and small hydrophilic peptides, will be eluted between Ala-Ala-Ala and Ala-Ser and Pro-Glu. The free amino acids, the small and very hydrophilic peptides and the hydro-

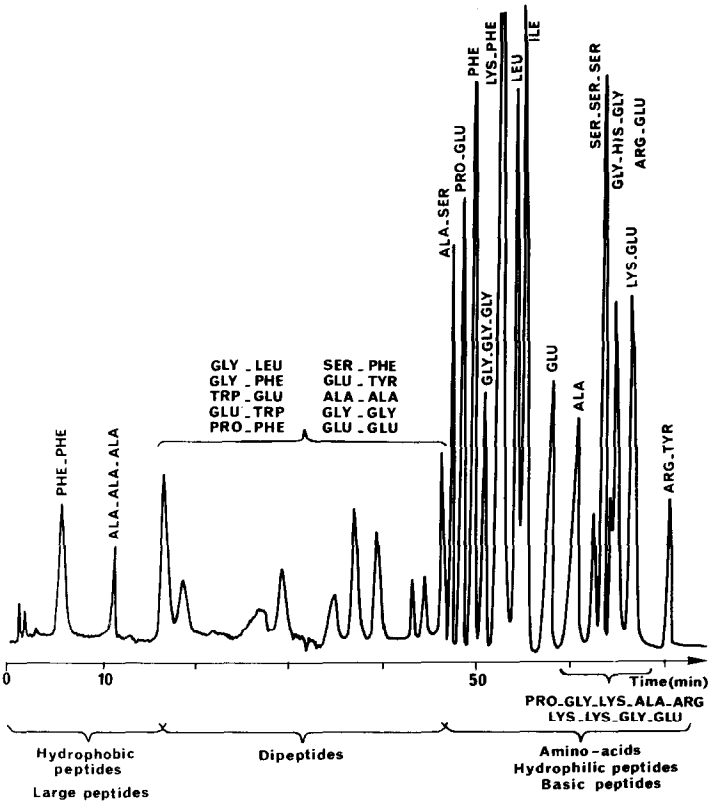

Fig. 8. Composite chromatogram of some peptides and amino acids. Conditions as in Fig. 7. Sample: 30 μl containing a few μg of the solutes.

philic and complexing peptides (those containing basic amino acids) will be eluted after Phe.

Fig. 9 shows the resulting chromatogram of some ovalbumin hydrolyzates, the same quantity (1 mg) of each being injected for the various chromatograms. The resolution is not sufficient to distinguish between the great variety of peptides produced by polyenzymatic degradations (c and d), and only the peak areas, shapes and relative proportions for each time zone give information on the quality of the enzymatic reactions and on the resulting mixtures. This type of gradient may be of

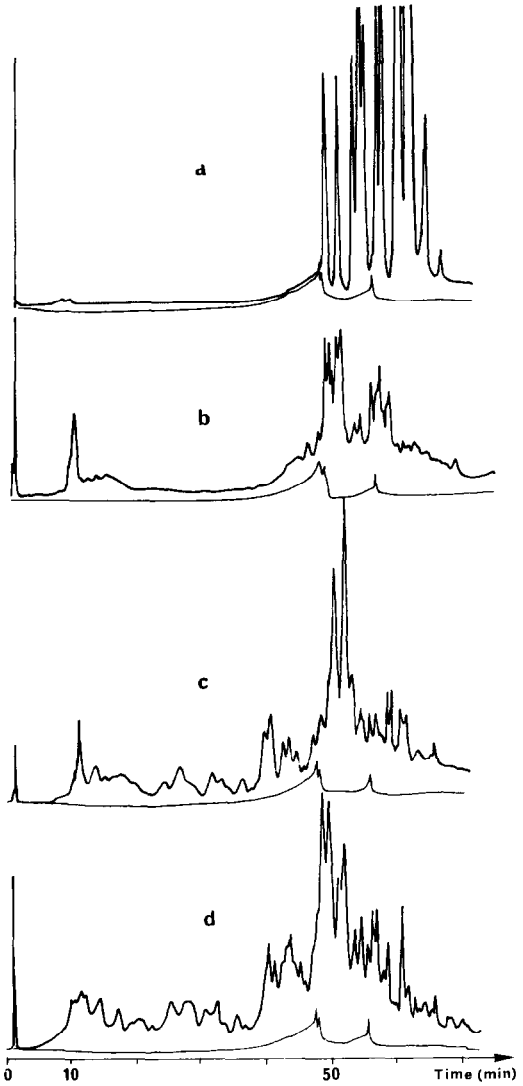

Fig. 9. Chromatograms of some ovalbumin hydrolyzates. Conditions as in Fig. 7. Sample: 30 μl containing: a, 1 mg of total hydrolyzate; b, 1 mg of pepsic hydrolyzate; c, 1 mg of di-enzymatic hydrolyzate; d, 1 mg of tri-enzymatic hydrolyzate. The lower tracing in each chromatogram is a blank.

interest when testing new conditions for an enzymatic degradation or checking hydrophilic properties of a peptide mixture or the purity of synthesized peptides. It must be noted that the elution order is roughly the opposite of the one found in reversed-phase chromatography (except for the basic peptides), *i.e.*, the more a peptide is retarded in reversed-phase chromatography, the easier it is eluted in ligand-exchange chromatography.

Class separation of dipeptides and amino acids from a complex mixture

The screening of protein hydrolyzate by ligand-exchange chromatography with gradient elution is useful for obtaining information on the variety and quality of a peptide mixture, but no quantitative estimation can be made because of the complexity of the chromatographic pattern. Previous studies[5] have shown that ligand-exchange chromatography is a good tool for obtaining information on the proportions of amino acids and small peptides in a complex mixture, the mobile phases being aqueous ammonia or buffered solutions, and the stationary phases organic polymers, like Sephadex or Chelex resins, one drawback is the rather long analysis time.

As we know that the retention time in ligand-exchange chromatography on copper(II)-modified silica gel is a result of both the normal-phase partition process and a ligand-exchange process with the ammonia–water–acetonitrile mobile phases, it becomes obvious that it will be necessary to work with mobile phases containing no acetonitrile if we want to use only the ligand-exchange process. Some attempts

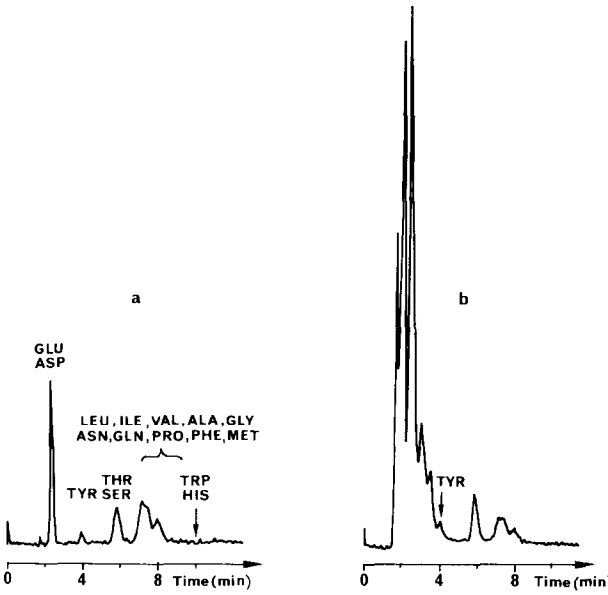

Fig. 10. Isocratic separation of amino acids from peptides. Column: 30 × 0.48 cm I.D. Packing: copper-(II)-modified LiChrosorb Si 60, d_p = 10 μm. Mobile phase: aqueous solution of 5 × 10^{-2} *M* TMED and 2 × 10^{-4} *M* Cu(II). Flow-rate: 1.5 ml/min. Pressure: 85 bar. Detection: UV at 325 nm. Sample: 30 μl containing: a (in μg): Glu (1.9), Asp (0.8), Tyr (0.6), Thr (0.7), Ser (1.6), Leu (1.6), Ile (1.3), Val (1.4), Ala (1.2), Gly (0.6), Asn (1.0), Gln (0.8), Pro (0.6), Phe (1.3), Met (0.9), Trp (0.2), His (0.4); b, 300 μg of an ovalbumin hydrolyzate.

have been made at elution with dilute aqueous ammonia solution from a copper(II)-modified silica gel column, and total separation of free amino acids, except Glu and Asp, from all the peptide fraction has been observed. However, despite the guard column, fritted end disks were often clogged by fines, due to the slow dissolution of silica gel.

We therefore decided to change the eluting base, as it has been observed earlier by Wehrli *et al.*[19] that primary, secondary and tertiary alkylamines attack the silicate structure less than do ammonia or quaternary ammonium hydroxide. In earlier work, we used tetramethylethylenediamine (TMED) as eluting base in order to use ligand-exchange chromatography on Cu(II)-silica gel in conjunction with *o*-phthalaldehyde (OPA) derivatization[20]. This tertiary diamine proved to be compatible with the stability of the copper(II)-modified silica gel, the very slow leakage of Cu^{2+} from the stationary phase being easily balanced by the use of a mobile phase containing Cu^{2+}.

We have tested mobile phases of TMED aqueous solutions of various concentrations with a Cu(II)(TMED)-modified silica gel column and guard column (see Experimental), and some results are reported here.

Separation of amino acids from peptides. Fig. 10 shows the chromatogram of a standard mixture of amino acids and the chromatogram of an ovalbumin hydrolyzate where the amino acid content had to be checked. The standard mixture was prepared according to the statistical amino acid composition of the hydrolyzate stud-

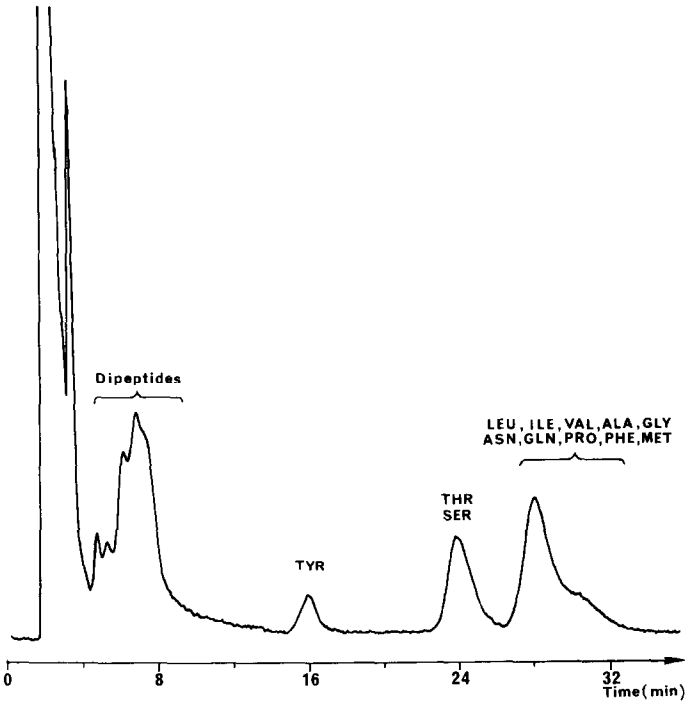

Fig. 11. Isocratic separation of amino acids and dipeptides from other peptides. Mobile phase: aqueous solution of 5×10^{-3} *M* TMED and 2×10^{-5} *M* Cu(II). Other conditions as in Fig. 10. Sample: 30 µl containing 100 µg of an ovalbumin hydrolyzate.

TABLE I

RETENTION TIMES FOR THE PEPTIDES TESTED

Peptide	*Retention time (min)*	*Peptide*	*Retention time (min)*	*Peptide*	*Retention time (min)*
Gly-Ser-Ala	1.8	Glu-Trp	2.7	Gly-Phe	6.7
Ala-Ala-Ala	1.8	Pro-Glu	3.2	Phe-Ser	6.9
Gly-Leu-Tyr	1.9	Met-Gly	5.4	Ala-Ser	7.1
Ser-Ser-Ser	1.9	Ser-Phe	5.7	Gly-Leu	9.8
Thr-Lys-Tyr	3.1	Phe-Phe	5.9	Pro-Phe	10
Glu-Tyr	2.1	Glu-Glu	6.2	Lys-Asp	23.2
Tyr-Glu	2.4	Gly-Gly	6.5	Ala-His	40
Trp-Glu	2.3	Ala-Ala	6.7	Arg-Tyr	40

ied. Except for Glu and Asp, which are eluted at the void volume with the peptide fraction, all amino acids are eluted in four groups, and are therefore very easy to quantify as a family. The whole peptide fraction is eluted before the Tyr peak, and the analysis time is less than 10 min.

Separation of dipeptides and amino acids from other peptides. Lowering the concentration of TMED (and of Cu^{2+}) in the mobile phase makes a new group emerge from the initial peak, as it may be seen in Fig. 11. As checked by injections of pure solutes, this emerging group is exclusively composed of dipeptides, except for basic ones, which are eluted later; the tested peptides are reported in Table I. Four time zones may be defined: from 0 to 3 min, elution of tri- and higher polypeptides; from 3 to 10 min, elution of dipeptides, except for the basic ones; the borderline (about 3 min) is composed of basic tri- and dipeptides having both an aromatic and acidic character (like Glu-Trp and Glu-Tyr); from 10 to 30 min, elution of free amino acids in three major peaks, and of some basic dipeptides (like Lys-Asp); after 30 min, elution of most of the basic dipeptides.

The dipeptide and amino acid fractions may easily be quantified by the injection of test mixtures, the composition of which is related to the hydrolyzate studied. Such an analysis has been performed in our laboratory and shown to agree with the results obtained by the Rothenbuhler method.

We have tested our methods for amino acid analysis and for amino acid and dipeptide analysis of ovalbumin hydrolyzates for weeks without any problems, and found them to be reliable and versatile.

CONCLUSIONS

The high selectivity of the ligand-exchange procedure, combined with the high efficiency obtained with the microparticulate copper(II)-modified silica gel, makes ligand-exchange chromatography a powerful tool.

The simplicity of the preparation of the column is attractive and the use of a guard column between the pump and the injector is a simple expedient for preventing the analytical column from slowly dissolving.

Combined 254-nm UV detection and gradient elution readily allow qualitative analysis of a complex polypeptide mixture, while isocratic elutions with aqueous

TMED mobile phases allow a simple and speedy quantitation of free amino acids and dipeptides in such hydrolyzates.

ACKNOWLEDGEMENTS

We are indebted to S. Desreumaux, Laboratoire Roger Bellon, for the gift of ovalbumin hydrolyzates and thank May Lahoud an Hien Pham Gia for helpful experimental assistance and comments.

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