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Chromatographic separation of mixed peptides from amino acids in biological digests with volatile buffers

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ABSTRACT

Two chromatographic methods, capable of separating mixed peptides from contaminating amino acids in biological digests, are described. Both methods involve separation on copper–Chelex resin, but each uses a different set of elution buffers. When separation method 1 was applied to a commercially available proteolytic digest of casein, the free amino acid content was reduced from 26.0% to 0.5%. With an enzymic digest of ^{14}C-labelled proteins derived from plant biomass, separation method 2 decreased the contaminating free amino acids from 20.3% to 1.9%. Since the separated peptides are eluted with volatile buffers, they are suitable as substrates for biological experiments.

INTRODUCTION

More and more research is being conducted into the metabolism of peptides across a wide range of biological systems. Such experimentation, especially that of a biochemical or nutritional nature, often requires procedures for the separation of complex mixtures of peptides either from their precursors, or from their catabolic products namely, amino acids. Several such chromatographic procedures exist, but they all possess disadvantages. Some, such as high-performance liquid chromatography (HPLC) [1], require elaborate and expensive laboratory equipment and are typically used to separate a limited number of peptides, often only one [2]. Similarly, flash chromatography, though relatively cheap [3], is mostly used to purify single peptides and indeed its ability to resolve complex mixtures of similar compounds has been questioned [4]. Most earlier separation procedures were based on the chelating properties of copper–Sephadex or copper–Chelex. Some of these concentrated on the separation of the amino acid fraction [5] and while others were capable of separating mixed peptides, the latter were always eluted in solutions and conditions, such as sodium tetraborate and at pH 11, that were quite unsuitable for use in biological experiments [5–7].

The aim of the present work was to prepare purified fractions of mixed peptides from protein digests using volatile solvents. The two methods reported here involve separations on columns of copper–Chelex resin, but each uses a different set of elution buffers. These methods were developed to separate peptidyl fractions from a commercially available proteolytic digest of casein (separation method 1) and also from an enzymic digest of ^{14}C-labelled proteins derived from plant biomass (separation method 2). Since the separated peptides are eluted with volatile buffers, they are suitable for subsequent biological applications, such as those of Cooper and Ling [8].

EXPERIMENTAL

Sources of peptides and amino acids

Two sources of mixed peptides and amino acids were used. One was a commercially available pancreatic digest of casein (Tryptone; Oxoid, London). The other was a ^{14}C-labelled mixture prepared by extracting water-soluble proteins from barley (*Hor-*

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deum vulgare cv. Kym or Gerbel) grown in a $^{14}CO_2$ atmosphere and digesting them with proteolytic enzymes based on the method of Cooper and Ling [8].

Preparation of copper–Chelex resin

Chelex 100 resin (50–100 mesh, sodium form; Bio-Rad, Richmond, CA, USA) was first washed with 1 *M* hydrochloric acid and then added to 0.16 *M* cupric sulphate solution; the mixture was adjusted to pH 2.5 with 5 *M* sodium hydroxide and stirred for 16 h. For use with the casein digest (separation method 1), the resultant copper–Chelex resin was washed with distilled water until the supernatant was colourless and then it was adjusted to pH 9.5 with 20 *M* ammonia solution. For the separation of the ^{14}C-labelled mixture (separation method 2), the copper–Chelex resin was washed with 0.1 *M* acetic acid until the eluent was colourless.

Separation of mixed peptides from amino acids

For method 1, a column of copper–Chelex resin (190 mm × 25 mm I.D.) was packed and washed with 100 ml 1 m*M* ammonia solution. Samples (50 mg of casein digest dissolved in 2.5 ml distilled water) were loaded onto the column, which was eluted sequentially with 100 ml 1 m*M* and 200 ml 5 *M* ammonia solution; the eluate was collected in 10.4-ml fractions. In method 2, a column of copper–Chelex resin (125 mm × 25 mm I.D.) was first washed with 100 ml 0.1 *M* acetic acid before samples (10 mg ^{14}C-labelled enzymic digest dissolved in 2 ml 0.1 *M* acetic acid) were loaded. The column was then developed with 240 ml 0.1 *M* acetic acid, 80 ml distilled water (to prevent the formation of ammonium acetate) and 480 ml 1 *M* ammonia solution; eluate fractions of 8.0 ml were collected. The elution flow-rate for both methods was approximately 60 ml/h.

Methods of analysis

The absorbances of eluate fractions from method 1 were measured at 280 nm. The radioactivity contents of fractions eluted from method 2 were measured with a liquid scintillant (Ecoscint; National Diagnostics, Manville, NJ, USA) and a scintillation counter (Model SL 30; Intertechnique SA, Plaisir, France) fitted with an external-standard channel-ratio facility to correct for quenching. Column effluent fractions that corresponded to a peak of either absorbance or radioactivity were pooled.

To remove any residual copper, each of the pooled groups was evaporated to dryness under reduced pressure at 37°C in a rotary evaporator, dissolved in 5 ml distilled water, loaded onto a column (60 mm × 7.5 mm I.D.) of Chelex 100 resin and eluted with five bed volumes of 0.01 *M* ammonia solution; the latter was removed from each pooled group by rotary evaporation.

Volumes of each pooled group were deproteinised with ice-cold picric acid [9]. Additional volumes derived from method 2 were acid-hydrolysed by refluxing in 6 *M* hydrochloric acid for 22 h [10]. The amino acid contents of both deproteinised and acid-hydrolysed samples were determined by cation-exchange chromatography and ninhydrin detection using a Locarte (London, UK) Model 5 analyser fitted with a Roseate data management system (Drew Scientific, London, UK). If asparagine or glutamine were present they would have been assayed as aspartic and glutamic acids respectively. Tryptophan concentrations were not measured in any of the samples. The peptide contents of the casein digest samples were estimated by the method of Lowry *et al.* [11]; the assay was calibrated with a standard solution of Tryptone. Peptidyl concentrations in the ^{14}C-labelled samples were calculated as the acid-hydrolysed values (total amino acids) minus those of the deproteinised, unhydrolysed supernatants (free amino acids).

RESULTS

The concentrations of free amino acids detected in the enzymic digest of casein before and after elution from a copper–Chelex column are shown in Table I. The proportion of free amino acids in the digest was calculated to be 26.0% (w/w), composed of these fifteen amino acids with especially high concentrations of free leucine and lysine. The relatively low concentration of tyrosine could not be accurately measured because it co-eluted with large peptidyl peaks.

The elution profile of the casein digest from a copper–Chelex column using separation method 1 is shown in Fig. 1. The first peak eluted (fractions 3–9) was found to contain 29.0 mg peptidyl material with only 0.14 mg free amino acid contamination, mainly in the form of glutamic acid (Table I). Table I also shows that the second peak (fractions 15–26)

TABLE I

FREE AMINO ACID CONTENTS BEFORE AND AFTER COPPER-CHELEX CHROMATOGRAPHY

Free amino acids of a commercially available enzymic digest of casein (mg per 50 mg sample loaded) and in elution fractions using separation method 1 and of an enzymic digest of ^{14}C-labelled proteins (mg per 10 mg sample loaded) and in elution fractions using separation method 2 as described in Experimental. – = Detection limit less than 0.005 mg amino acid. * = not determined because of co-elution with peptides, see Results.

Amino acid	Method 1			Method 2		
	Casein digest	Fractions 3–9	Fractions 15–26	^{14}C-labelled mixture	Fractions 6–21	Fractions 54–65
Asp	–	–	–	0.35	0.30	–
Thr	0.37	–	0.12	0.13	0.12	–
Ser	0.50	–	0.15	0.20	0.18	–
Glu	0.20	0.07	0.01	0.32	0.28	–
Pro	–	–	–	0.07	0.04	–
Gly	0.07	–	0.05	0.54	0.48	0.01
Ala	0.24	–	0.16	0.27	0.25	–
Cys	0.13	–	0.01	–	–	–
Val	0.70	–	0.45	–	–	–
Met	0.75	0.02	0.33	–	–	–
Ile	0.62	0.02	0.28	–	–	–
Leu	2.81	0.03	1.55	0.08	–	–
Tyr	*	–	0.08	*	–	*
Phe	0.96	–	0.47	*	–	*
His	0.31	–	0.19	–	–	–
Lys	3.51	–	1.64	0.06	–	0.06
Arg	1.82	–	0.03	–	–	–

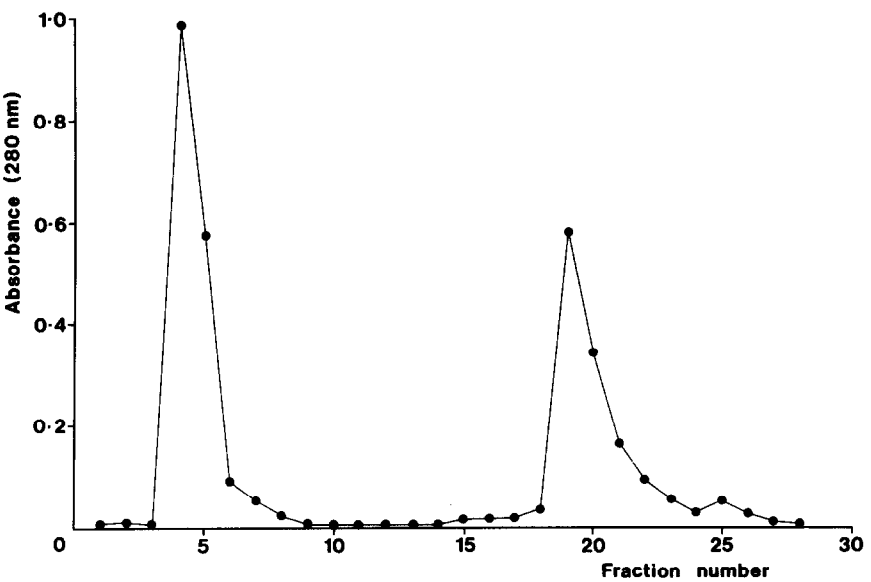

Fig. 1. Elution profile from a copper–Chelex resin column of an enzymic digest of casein (sample size, 50 mg dissolved in 2.5 ml water) eluted according to separation method 1.

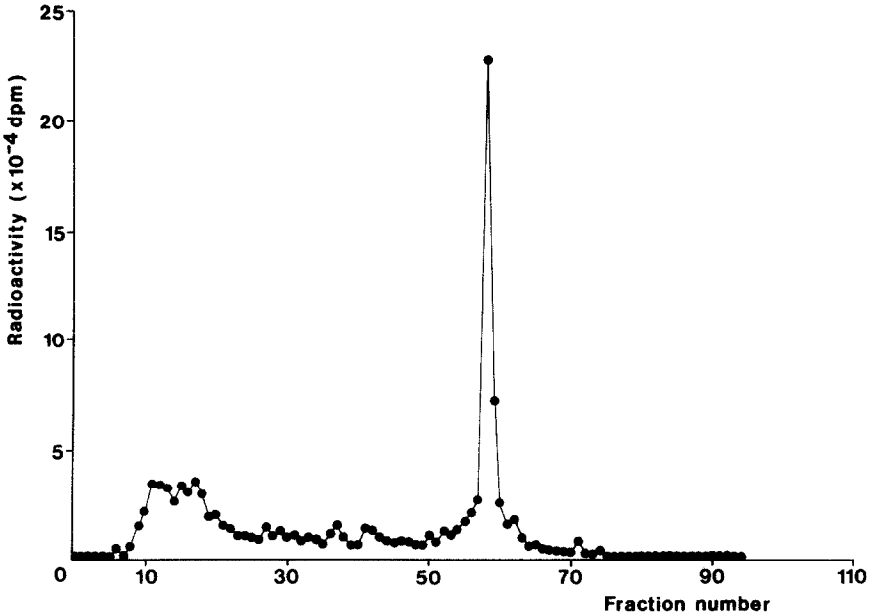

Fig. 2. Elution profile from a copper–Chelex resin column of an enzymic digest of ^{14}C-labelled proteins (sample size, 10 mg dissolved in 2 ml 0.1 *M* acetic acid and containing $1.19 \cdot 10^6$ dpm) eluted according to separation method 2.

contained a large amount (5.52 mg) of free amino acids together with 9.2 mg mixed peptides.

The free amino acid composition of the ^{14}C-labelled mixture is shown in Table I; the eleven amino acids that were detected accounted for 28.1% (w/w) of the sample. Values for free tyrosine and phenylalanine are not included because they co-eluted with a number of peptides which interfered with their accurate estimation, nevertheless their concentrations appeared to be negligible.

The elution pattern of radioactivity of the ^{14}C-labelled mixture of amino acids and peptides from a copper–Chelex column using separation method 2 is shown in Fig. 2. Analysis of the first peak (fractions 6–21) showed that it contained 1.65 mg free amino acids and 0.9 mg peptides, whereas the second peak (fractions 54–65) contained 3.6 mg peptides, but only 0.07 mg amino acids.

DISCUSSION

The two separation methods, based on copper–Chelex chromatography described here, have been successfully used to prepare fractions of mixed peptides by removing the majority of contaminating free amino acids from biological samples containing the two species. Free amino acid contamination has been reduced from 26.0% to 0.5% in an enzymic digest of casein using separation method 1 and from 20.3% to 1.9% in a mixture of ^{14}C-labelled peptides and amino acids derived from an enzymic digest of plant proteins using method 2.

The difference in effectiveness of the two separation methods apparently depends upon the predominant amino acids contaminating the peptides. When a standard solution of amino acids (AA-S-18; Sigma, Poole, UK) was subjected to separation by method 1, aspartic and glutamic acids were eluted in fractions 4–9, whereas the other amino acids appeared in fractions 15–26. Furthermore, when a sample of casein digest was eluted under the conditions of method 2, separations were most unsatisfactory; the first peak was found to contain 4.17 mg free amino acids and 9.8 mg peptides, while the second peak contained 3.22 mg free amino acids, composed mostly of free phenylalanine, histidine and lysine, with 29.8 mg peptide material.

Method 1 therefore seems to be more efficient at removing the basic amino acids, whereas method 2 is more efficient when acidic amino acids are pres-

ent. Since the casein digest contained comparatively low concentrations of free glutamic acid and no free aspartic acid, method 1 is the appropriate choice for this mixture. On the other hand, the ^{14}C-labelled mixture contained relatively large concentrations of these acidic amino acids and low concentrations of the basic amino acids, so separation method 2 is the more suitable procedure.

Comparisons of the mechanistically similar copper–Sephadex separation method of Rothenbühler *et al.* [7] with the copper–Chelex procedure described here as method 2, showed the former to be far less effective; using the ^{14}C-labelled mixture, the peptide fraction eluted from the copper–Sephadex column was still contaminated by as much as 20% free amino acids. And in addition, the problem of the removal of tetraborate remained. Furthermore, the volatile eluent, ammonia solution, used in method 2 could not be used to elute copper–Sephadex columns as the copper ions would be removed and precipitated at such a high pH.

Using methods 1 and 2, recoveries of the original peptidyl material were 78% for the casein digest and 45% for the ^{14}C-labelled mixture. Incomplete recoveries of peptides appear to be typical of many chromatographic procedures; for example, Rothenbühler *et al.* [7], using their copper–Sephadex method, stated that "the recovery for most peptides is higher than 80%" and a similar value has been reported for an HPLC method [12]. The poor recovery of peptides from the ^{14}C-labelled mixture separated by method 2 was investigated in an additional trial. When fractions 22–53 were collected, combined and re-eluted through the copper–Chelex column, the distribution of radioactivity altered so that when the re-eluted fractions 54–65 were combined with the original 54–65 fractions, a recovery of 76% peptidyl material was obtained. Thus improved yields of ^{14}C-labelled purified mixed peptides may be obtained, if required, by this procedural addendum.

A major advantage of these reported methods is that they require only simple, cheap laboratory equipment, as opposed to HPLC procedures. Furthermore, since the peptidyl fractions are eluted with acetic acid, water or ammonia solutions, which are easily removed by evaporation under reduced pressure, they may be used as substrates in subsequent biological experiments [8]. These procedures thus overcome the problems of elution with non-volatile buffers, such as sodium tetraborate, in previously reported copper–Chelex [6] and copper–Sephadex [5,7] methods. These particular advantages may also apply to procedures using flash chromatography, but as yet no comparable methods have been published using such a system and doubts have been expressed concerning its potential to resolve such complex mixtures [4]. Finally, in addition to their usefulness in purifying preparative amounts of peptidyl substrates, the copper–Chelex methods reported here may be of benefit in pre-processing crude samples prior to HPLC analysis.

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