Journal of Chromatography, 200 (1980) 185–188
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,110

THE ISOLATION OF METHIONINE AND ETHIONINE BY SILVER LIGAND CHROMATOGRAPHY AND APPLICATION TO METHIONINE-CONTAINING PEPTIDES

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(Received July 8th, 1980)

SUMMARY

A simple procedure in which silver ligand chromatography is used to isolate methionine and ethionine from amino acid mixtures is described. This facilitates the estimation of ethionine by amino acid analysis. The separation of methionine-containing peptides from an enzymic hydrolysate of a protein is also reported, and its use in ordering cyanogen bromide fragments is suggested.

INTRODUCTION

Ethionine is a powerful carcinogen[1], and hence we wanted to determine whether it was present in some foodstuffs. In the chromatographic method of Spackman *et al.*[2] for the separation of amino acids in protein hydrolysates[2], ethionine is not resolved from leucine. We therefore developed a way of separating methionine and ethionine from other neutral and acidic amino acids, such that an amino acid analyser could then readily be used to detect and measure ethionine. The method is based on the formation of a ligand between silver ions and sulphur. The silver ions are attached to a cation-exchange resin, and the sample is passed down the column, which retains the sulphur-containing amino acids. Methionine and ethionine can be eluted by lowering the pH of the eluting solution. The method is also applicable to the isolation of peptides containing methionine.

EXPERIMENTAL AND RESULTS

Column preparation

The standard method of preparing columns was as follows: a column (15 × 0.6 cm) of Amberlite CG-50 [chromatographic grade, type I (BDH, Poole, Great Britain)] was washed successively with 0.1 *M* NaOH, water, 0.1 *M* acetic acid

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and water until again neutral. The column was then loaded with Ag^+ by passing $AgNO_3$ (0.1 *M*, 50 ml) through it and eluting the Ag^+ with water; the eluent was tested for Ag^+ with Cl^-. The column was protected from light by wrapping it in aluminium foil.

Sample preparation included the removal of Cl^- by the dropwise addition of $AgNO_3$ (0.1 *M*) until no further precipitation of AgCl was apparent, centrifugation, neutralisation (pH 5–7) of the supernatant with dilute NaOH or acetic acid, and dilution to contain *ca.* 1 mg of amino acids per 10 ml of solution. After application of the sample, the column was washed with 20 ml of water.

Protein hydrolysates

In the case of protein hydrolysates or other amino acid mixtures, sulphur-containing and basic amino acids were retained by the column. These amino acids and the Ag^+ were eluted by acetic acid (0.1 *M*, 20 ml). The Ag^+ was removed as AgCl by centrifugation after the dropwise addition of 6 *M* HCl. Samples were then analysed using an amino acid analyser (Model 120 C; Beckman Instruments, Palo Alto, CA, U.S.A.). There was a quantitative recovery of methionine and ethionine, compared with samples not subjected to the silver ligand chromatography. Oxidation to sulphoxides was not evident, but the addition of thiodiglycol (0.05 ml per 10 ml of solution) during chromatography on the silver ligand column could be used if thought necessary. Cysteine and cystine were not recovered, but were probably lost due to the formation of insoluble silver derivatives. Carboxymethylcysteine was not retained on the silver ligand column. The sulphones produced by performic acid oxidation of methionine and ethionine were not retained on the column, and this phenomenon provides the basis for selective isolation of peptides containing methionine or ethionine.

Peptides containing methionine

Substilisin BPN′ (Calbiochem, Los Angeles, CA, U.S.A.) was heat denatured (20 mg protein per 2 ml of H_2O; 100°C; 5 min) before digestion with subtilisin BPN′ (2% (w/w) of protein in 0.5% (w/v) NH_4HCO_3, pH 8; 37°C; 4 h) and lyophilized. The peptides were dissolved in water and applied to the silver ligand column. Acidic or neutral peptides containing no methionine passed through the column, whereas peptides containing methionine or basic residues were retained. These retained peptides were eluted by acetic acid (50%, v/v) and, after removal of Ag^+ and lyophilization, were subjected to performic acid oxidation[3] and re-lyophilized. When this mixture was passed down a silver ligand column, the peptides containing oxidised methionine were no longer retained and were thus separated from the basic peptides. Aliquots of the mixture and of the eluents at each stage were subjected to amino acid analysis in order to follow the separation. Peptide mapping and analysis showed that all of the peptides contained methionine and were those that could be expected from a subtilisin digest of subtilisin BPN′.

Methionine infusion into sheep

The method was first developed to examine the suggestion of Miller[4] that ethionine may be produced in the caecum and large intestine of ruminants. This possibility was raised because the synthesis of ethionine in bacteria, such as

Escherichia coli, is enhanced by exposure to increased concentrations of methionine[5]. Any ethionine so produced could be absorbed from the gut and act as a powerful carcinogen[1]. In recent years, a process has been developed to protect feed protein from ruman microbial digestion in ruminants[6], and this may result in increased concentrations of amino acids reaching the large intestine. Thus, the chances of ethionine formation are enhanced when "protected" protein is fed. In order to test this hypothesis L-[1-^{14}C]methionine (Radiochemical Centre, Amersham, Great Britain) was infused into the duodenum in two lactating sheep. In the first experiment, methionine (240 mg, 150 μCi) was infused over a period of 5 min. In the second experiment, non-radioactive methionine was infused at the rate of 1.9 g/day for 7 days before the introduction of [^{14}C]methionine (100 μCi) and at the rate of 10.8 g/day for 14 days afterwards. Samples of portal blood, venous blood and milk from both sheep were examined for the presence of ethionine or radioactivity eluted from the amino acid analyser with the same retention volume as ethionine. Radioactivity was detected prior to mixing with ninhydrin by using an anthracene-packed flow-cell in a scintillation counter (Nuclear-Chicago 720 Series, Chicago, IL, U.S.A.). In the second experiment, samples of heart muscle, liver and kidney were obtained at the end of the infusion. However, there was no evidence of any ethionine being produced in either experiment.

Artifactual production of ethionine

Ethionine was observed as an artifact in acid hydrolysates of tissues that had been frozen in the presence of ethyl acetate. It was found that ethionine could be produced in appreciable amount by the reaction of homocysteine with ethyl acetate under the hydrolysis conditions used (6*M* HCl, evacuated sealed tubes, 110°C, 22 h). The formation of a thiol ether from a primary alkyl ester had not been expected[7].

DISCUSSION

Silver ligand chromatography has been used for the separation of compounds on the basis of the number, position and configuration of their double bonds[8–10] and for the separation of nitrogen-containing compounds[11]. Silver is also known to bind to sulphur and here has been successfully applied to the separation of methionine and ethionine from neutral amino acids. These separations and the non-retention of oxidized methionine peptides can be explained in terms of Pearson's unifying hypothesis of "hard and soft acids and bases"[12]. In some applications of metal ligand chromatography, it may be important to retain the metal on the support[10]; however, in our case this was not considered necessary, and silver was removed from fractions by precipitation as the chloride.

The gross separation of methionine and ethionine from other neutral amino acids that we achieved enabled quantitation by conventional ion-exchange chromatography. Similarly, peptide mapping was simplified by the initial isolation of a methionine-containing fraction with the ligand columns. The chromatographic separation of mixtures of other sulphur-containing compounds should be possible using soft metal ligands.

An alternative procedure for isolating methionine-containing peptides which would avoid the need for the oxidation used above, would be to pass the enzymic

digest (plus thiodiglycol to protect against oxidation) down a column of CG-50 resin to remove peptides containing basic residues. Silver-loaded CG-50 would then collect only the methionine-containing peptides, which would be eluted with acetic acid, and there would be the possibility of resolution of these peptides. Methionine and ethionine could be isolated from basic as well as from neutral and acidic amino acids by such a procedure.

Subtilisin BPN' provided a most favourable example for the isolation of all methionine-containing peptides from a protein[13], because none of the peptides from the subtilisin digest contained a basic residue, which would have been retained on the silver column after the performic acid oxidation. For other proteins, analysis of the fractions at each stage would indicate whether there was a basic residue close to a methionine. In such instances it may be possible to overcome the problem by succinylation[14] of lysyl residues and reaction of arginine with glyoxal[15]. If a tryptic digest were to be examined, the terminal basic residues could be removed with carboxypeptidase B prior to loading the digest on the silver column.

The usefulness of the selective isolation of methionine-containing peptides to enable the ordering of cyanogen bromide fragments during protein sequencing, has been discussed by Shechter *et al.*[16] as a use of their polymeric reagent. The present silver ligand column would appear to be simpler to prepare and use.

ACKNOWLEDGEMENTS

We wish to thank Dr. M. W. Simpson-Morgan for carrying out the necessary surgery, and Mrs. Nancy Aldrich and Mr. B. J. Barancewicz for skilful technical assistance. We also wish to thank Dr. D. D. Perrin for useful discussions.

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