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## Note

# Simultaneous determination of L-homoserine and L-homoserine lactone by reversed-phase liquid chromatography in acid hydrolysates of proteins after cyanogen bromide treatment

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Oxidation of L-amino acids in proteins is increasingly considered to be of importance in ageing and inflammatory processes. One critical amino acid is L-methionine (Met), which is easily oxidized to L-methionine sulphoxide [Met(O)] [1–4]. To determine the extent of Met oxidation in proteins from diseased or altered sites, both Met and Met(O) have to be measured by amino acid analysis. During hydrolysis of proteins with azeotropic hydrochloric acid, however, Met(O) is partially decomposed to Met. Consequently, the direct determination of Met and Met(O) in the acid hydrolysates is subject to considerable error [5]. To circumvent these difficulties, the proteins are modified by reaction with cyanogen bromide (CNBr) prior to hydrolysis [1,6,7]. During this procedure, non-oxidized Met residues are quantitatively converted to L-homoserine lactone (Hsl) which exists in a pH-dependent equilibrium with L-homoserine (Hse). Oxidized Met residues are not modified by reaction with CNBr [6,7]. In conventional amino acid analysis, Hse runs between L-serine

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and L-glutamic acid, whereas the lactone appears in the region between ammonia and arginine [6,8–10]. As the colour yield of Hse after reaction with ninhydrin is much lower than that of Hsl [8,9], quantitation of the originally non-oxidized Met as the sum of Hse and Hsl in hydrolysates of CNBr-digested proteins is inaccurate.

In this paper, we describe a new method for the determination of total Hse (Hse plus Hsl) by reversed-phase high-performance liquid chromatography (RP-HPLC) using precolumn derivatization with *o*-phthaldialdehyde (OPA) at pH 10.4. Under the conditions used for precolumn derivatization, Hsl is completely converted to Hse, which allows the simultaneous determination of Hse plus Hsl and residual Met by RP-HPLC in CNBr-digested proteins without artifacts.

## EXPERIMENTAL

### *Materials*

L-Amino acid calibration mixture, OPA reagent solution, sample diluent buffer (1.0 *M* potassium borate, pH 10.4) and CNBr were from Pierce (Rockford, IL, U.S.A.), acetonitrile (HPLC gradient grade) and tetrahydrofuran (HPLC gradient grade) from Baker (Gross-Gerau, F.R.G.), Hse, Hsl and human $\alpha$-1-proteinase inhibitor ($\alpha$-1-PI) from Sigma (Taufkirchen, F.R.G.) and all other chemicals from Merck (Darmstadt, F.R.G.). Human bronchoalveolar lavage (BAL) fluids were obtained by standardized washing of the right middle lobe with 5 × 20 ml of sterile 0.9% (w/v) saline during fiberoptic bronchoscopy. The fluid recovery was approximately 50–60%.

### *Cyanogen bromide treatment of proteins*

Portions of 100 $\mu$g of $\alpha$-1-PI or BAL fluid proteins, dialysed against water for 3 h at 4°C, were dissolved in 840 $\mu$l of 75% (v/v) formic acid (nitrogen-saturated). Conversion of Met to Hse was performed by addition of 60 $\mu$l of 3 *M* CNBr in acetonitrile. The reaction mixtures were kept under nitrogen saturation in darkness at room temperature. After incubation for 24 h the samples were lyophilized twice.

### *Hydrolysis*

Native $\alpha$-1-PI and the CNBr-digested protein were subjected to hydrolysis with doubly distilled 6 *M* hydrochloric acid in the presence of 5 m*M* 2-mercaptoethanol or dithioerythritol at 110°C under vacuum. Under these conditions, Met(O) is quantitatively recovered as Met [7].

### *RP-HPLC analysis*

RP-HPLC analysis was performed on a 3-$\mu$m Spherisorb ODS-2 column (250 mm × 4.6 mm I.D.) from Grom (Herrenberg, F.R.G.). The assays were

carried out with an HPLC analyser from LKB (Freiburg, F.R.G.) at a flow-rate of 1.0 ml/min using an autosampler for automatic precolumn derivatization with OPA. For precolumn derivatization, the lyophilized hydrolysates were dissolved in 0.2 *M* potassium borate buffer (pH 10.4) containing 1–10 nmol of each amino acid and incubated for 60 min at room temperature. A 30-$\mu$l aliquot of each hydrolysate was mixed with 10 $\mu$l of OPA reagent and a 15-$\mu$l portion of the reaction mixture was immediately applied to the HPLC column. Elution was performed by a gradient: solvent A was a mixture of 960 ml of 12.5 m*M* sodium phosphate buffer (pH 7.25), 20 ml of acetonitrile and 20 ml of tetrahydrofuran and solvent B a mixture of 500 ml of 12.5 m*M* sodium phosphate buffer (pH 7.25) and 500 ml of acetonitrile, and the portion of solvent B in the gradient was increased from 10% at the beginning of the run to 33% (v/v) within 15 min, maintained at 33% (v/v) for 12 min and increased to 77% (v/v) within 17 min. The column was subsequently regenerated with solvent B for 3 min and equilibrated for 10 min with a mixture of solvents A and B (9:1, v/v). Peaks were detected with a Hitachi F 1000 fluorescence analyser (excitation at 330 nm and emission at 450 nm) and evaluated with a Shimadzu integrator.

*Conversion of homoserine lactone to homoserine at alkaline pH*

For studies of the ring opening of Hsl to Hse at alkaline pH, Hsl was incubated in 0.2 *M* potassium borate buffer (pH 10.4) at room temperature. After 1, 2, 5, 10, 30 and 60 min, samples were taken and the pH immediately changed to 3.0 by 1:100 (v/v) dilution with 10 m*M* sodium citrate buffer (pH 3.0). Aliquots of 1 ml were applied to an SP-Sephadex column (1 cm$\times$0.7 cm, I.D.) equilibrated with 10 m*M* sodium citrate buffer (pH 3.0). Hse was quantitatively eluted by washing the column with 5 ml of the equilibrating buffer. Hsl, which is completely retained under these conditions, was subsequently eluted with 5 ml of 10 m*M* sodium citrate buffer (pH 6.0). The fractions were then analysed by RP-HPLC.

## RESULTS AND DISCUSSION

*RP-HPLC of homoserine and homoserine lactone*

After precolumn derivatization with OPA, Hse and Hsl were analysed on a reversed-phase column to determine their chromatographic behaviour. The two compounds were eluted at identical retention times (Fig. 1a and b). A mixture of Hse and Hsl appeared as a single, sharp peak (result not shown). Hse was well separated from the adjacent peaks, i.e., L-histidine and L-glycine (Fig. 1c). Additionally, mixtures of equimolar concentrations of Hse and Met or Hsl and Met were analysed to calculate the relative peak area for Hse (ratio of Hse to Met) and the lactone (ratio of Hsl to Met). In the separation system described here, both Hse and Hsl had relative peak areas of 0.99, indicating

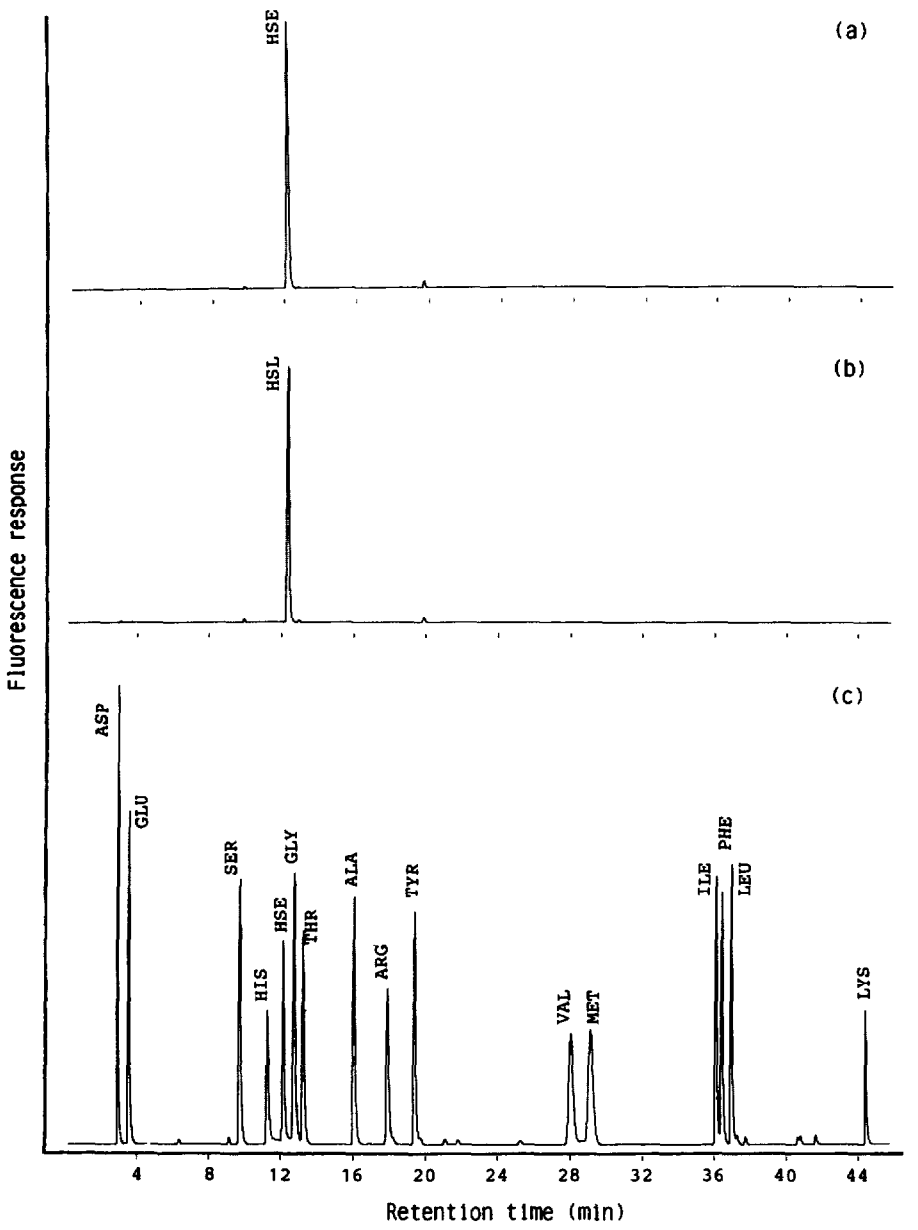


Fig. 1. RP-HPLC trace for amino acids after precolumn derivatization with OPA at pH 10.4. All samples were incubated for 60 min in 0.2 *M* potassium borate buffer (pH 10.4) at room temperature prior to modification with OPA. For details, see Experimental. (a) 112 pmol of Hse; (b) 112 pmol of Hsl; (c) 115 pmol of an amino acid calibration mixture plus 90 pmol of Hse. The small peaks are impurities.

identical fluorescence recoveries. Hence it appears that Hsl is converted to Hse under the alkaline conditions necessary for precolumn derivatization with OPA. Ring opening of Hsl during precolumn derivatization seems to be complete, as virtually all the substance elutes as a single peak (Fig. 1b). The small peak at 19.8 min amounted to less than 3% of the major peak and can therefore be neglected. This second peak is more likely to be contamination than unconverted Hsl, as analysis of Hse showed the same second peak at 19.8 min (cf. Fig. 1a).

Conversion of Hsl to Hse is assumed to occur after dissolution of the sample in 0.2 *M* potassium borate buffer (pH 10.4), which is used for precolumn derivatization with OPA. We therefore studied the time course of ring opening of Hsl in this buffer at 25°C. Samples were taken at different times, followed by an immediate change in the pH to 3.0 to stop further hydrolysis of the lactone. Complete conversion of Hsl to Hse in 0.2 *M* potassium borate buffer (pH 10.4) was found to occur within 30 min. We suggest an incubation time of 60 min for conversion of Hsl to Hse in 0.2 *M* potassium borate buffer (pH 10.4) prior to modification with OPA.

Opening of the Hsl ring has already been demonstrated by Offord [11], using triethylamine buffer (2%, w/v), adjusted to pH 10.0 with solid carbon dioxide. He found that this process occurs at room temperature within 20–30 min. Jones and Gurd [10] treated CNBr–glucagon with aqueous 0.2 *M* triethylamine (pH 9.5) for 20 min at room temperature for ring opening of Hsl.

*Stability of homoserine during acid hydrolysis*

Free Hse was subjected to hydrolysis with azeotropic hydrochloric acid to study its stability under these conditions (for details, see Experimental). Met, present at the same concentration as Hse, was used as an internal standard. To ensure complete recovery of Met during acid hydrolysis, dithioerythritol was added as a reducing agent. The relative peak area of Hse (ratio of Hse to Met) determined after hydrolysis for 48 h was found to be 1.00, in comparison with 0.99 for the unhydrolysed sample, indicating that free Hse is completely recovered after acid hydrolysis.

*Determination of homoserine in cyanogen bromide-modified α-1-PI*

We were interested to see whether unoxidized Met can be correctly determined in CNBr-modified proteins by quantitation of Hse by RP-HPLC. We used human α-1-PI, which contains nine Met residues [12], as a test substance. After treatment of the protein with CNBr, aliquots of the sample were hydrolysed for different times with 6 *M* hydrochloric acid and analysed by RP-HPLC (Table I). The molar percentages of Hse and residual Met were calculated for each hydrolysis time. No decrease in Hse was observed with increasing hydrolysis times up to 96 h, which confirms the stability of Hse during acid hydrolysis. The average value of 0.21 mol-% for the residual Met found in the

TABLE I

AMINO ACID COMPOSITION OF THE CNBr-TREATED α-1-PI ESTABLISHED BY RP-HPLC AFTER PRECOLUMN DERIVATIZATION WITH OPA

| Amino acid[a] | Molar percentage | | | | |
|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 96 h | Average or extrapolated value |
| Asp | 12.47 | 12.72 | 12.91 | 13.16 | 12.82[b] |
| Glu | 13.59 | 14.23 | 14.33 | 14.33 | 14.12[b] |
| Ser | 5.23 | 3.81 | 2.98 | 2.22 | 7.00[c] |
| His | 3.28 | 3.47 | 3.61 | 3.65 | 3.50[b] |
| Gly | 7.31 | 7.02 | 6.79 | 7.47 | 7.15[b] |
| Thr | 7.19 | 6.82 | 6.23 | 5.74 | 7.80[c] |
| Ala | 7.61 | 7.43 | 7.56 | 7.82 | 7.61[b] |
| Arg | 2.87 | 2.84 | 2.87 | 2.82 | 2.85[b] |
| Tyr | 2.31 | 2.34 | 2.42 | 2.51 | 2.40[b] |
| Val | 7.05 | 7.49 | 7.63 | 7.79 | 7.79[d] |
| Ile | 4.82 | 5.05 | 5.21 | 5.28 | 5.28[d] |
| Phe | 6.43 | 6.69 | 6.89 | 6.98 | 6.75[b] |
| Leu | 11.58 | 11.58 | 11.83 | 11.76 | 11.69[b] |
| Lys | 8.24 | 8.49 | 8.90 | 8.46 | 8.52[b] |
| Hse | 2.22 | 2.21 | 2.24 | 2.25 | 2.23[b] |
| $Met_{res}$[e] | 0.24 | 0.20 | 0.21 | 0.21 | 0.21[b] |
| $Met_{tot}$ (Hse + $Met_{res}$) | 2.46 | 2.41 | 2.45 | 2.46 | 2.44[b,f] |

[a]The amino acids Pro, Cys and Trp were not determined.
[b]Average value.
[c]Extrapolated to zero time.
[d]Extrapolated to 96 h hydrolysis.
[e]Residual Met after CNBr digestion. The residual Met after reduction of α-1-PI with 1 *M* 2-mercaptoethanol for 120 h at 56 °C prior to CNBr digestion was determined to be < 0.025 mol-%.
[f]Total Met in the native α-1-PI was determined to be 2.40 mol-% by separate amino acid analysis.

CNBr-digested protein is believed to be identical with the amount of oxidized Met originally present in the α-1-PI. This was confirmed with an α-1-PI preparation that had been reduced with 1 *M* 2-mercaptoethanol prior to modification with CNBr. The CNBr digest of the reduced protein contained less than 0.025 mol-% of residual Met (Table I).

The sum of Hse and residual Met in the CNBr-digested protein was calculated to be 2.44 mol-%. This value is close to the total Met content of 2.40 mol-% that we determined by separate amino acid analysis of the native α-1-PI (Table I). The value for Met calculated from sequence analysis data is 2.50 mol-% [12]. Our results show clearly that Hsl plus Hse in acid hydrolysates of CNBr-digested proteins can be detected precisely in a single peak by RP-HPLC

TABLE II

MET(O) CONTENT OF THE TOTAL PROTEIN IN BRONCHOALVEOLAR LAVAGE (BAL) FLUIDS FROM HEALTHY NON-SMOKING VOLUNTEERS AND PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS (IPF)

| Study population | Met(O) content [mol Met(O)/mol Met] |
|---|---|
| *Healthy volunteers (non-smokers)* | |
| No. 1 | 0.042 |
| No. 2 | 0.030 |
| No. 3 | 0.015 |
| No. 4 | 0.049 |
| No. 5 | 0.034 |
| No. 6 | 0.021 |
| No. 7 | 0.028 |
| Mean ± S.D. | 0.031 ± 0.012 |
| *Patients with IPF (non-smokers)* | |
| No. 1 | 0.145 |
| No. 2 | 0.678 |
| No. 3 | 0.362 |
| No. 4 | 0.135 |
| No. 5 | 0.218 |
| No. 6 | 0.157 |
| No. 7 | 0.332 |
| Mean ± S.D. | 0.290 ± 0.190 |

after precolumn derivatization with OPA at pH 10.4, which allows an accurate determination of total unoxidized Met in the proteins. The amount of Met(O) can be measured simultaneously as residual Met in the same system. Hence the molar ratio between oxidized and unoxidized Met [Met(O)/Met] can be determined easily and used as a parameter for the oxidative modification of proteins.

*Analysis of proteins from BAL fluids for Met(O)*

The method described was first applied to BAL fluid proteins from healthy non-smoking volunteers (controls) and patients with idiopathic pulmonary fibrosis (IPF). There was a significant increase in the Met(O)/Met ratio of BAL fluid proteins from IPF patients when compared with the control samples (Table II). This finding reflects oxidative processes in the lungs of IPF patients and is consistent with the data reported by Cantin et al. [13]. They found that BAL fluids from IPF patients contained increased levels of the enzyme myeloperoxidase, which modulates oxidant-mediated injury of the lung parenchymal cells.

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