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SHORT COMMUNICATION

Cysteine Racemization in Peptide Synthesis: A New and Easy Detection Method

FRANK SIEDLER, ELISABETH WEYHER and LUIS MORODER

Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany

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Abstract: A new method has been developed for the rapid determination of D-cysteine contents in synthetic peptides. It is based on the reduction of cystine residues, when present, with tris-alkylphosphines, selective derivatization of the cysteine residues with 4-vinylpyridine, followed by acid hydrolysis of the (4-pyridylethyl)cysteine–peptides. Baseline enantiomeric resolution of the D,L-*S*-β-(4-pyridylethyl)cysteine, and thus quantification of D-enantiomer contents at levels $\leq 1\%$, is easily achieved by capillary zone electrophoresis exploiting the host–guest complexation principle with crown ethers or by gas chromatography on chiral glass capillary columns upon conventional derivatization of the hydrolysate. The acid-stability of the (4-pyridylethyl)cysteine derivative prevents racemization via thiazoline intermediates and allows for standardization of the acid hydrolysis-dependent racemization.

Keywords: peptide synthesis; cysteine; racemization; enantiomeric resolution; capillary electrophoresis; gas chromatography

INTRODUCTION

Independent of the thiol-protecting groups used in the synthesis of cysteine–peptides, the easy racemization of this residue by Cα-proton abstraction has been recognized as a serious problem since the early days of peptide chemistry. It has become even more serious in the solid-phase synthesis of cysteine–peptides since racemization occurring during the esterification of amino acids to hydroxymethyl polymers is a recognized problem [1] and particularly prevalent in the case of cysteine [2, 3]. Moreover, it is well established that cysteine esters are unusually prone to racemization via the Cα-proton abstraction mechanism in the presence of excesses of amines [4, 5]. Correspondingly, a high degree of racemization of cysteine esterified to the resin has been observed during peptide chain elongation by the Fmoc/tBu strategy, i.e. on repeated exposure to piperidine [6, 7]. Thereby, the type of both the thiol-protecting group and the ester linkage to the resin was found to affect the rate of epimerization significantly. Additionally, an unexpectedly high degree of racemization was detected even for urethane-protected cysteine derivatives when coupling reactions were performed with the commonly used acylating agent TBTU/HOBt in the presence of DIEA as an auxiliary base [8–11]. When condensations were performed in the absence of a base, e.g. via the pentafluorophenyl ester [8, 9] or via the symmetric anhydride [10, 11], this racemization was almost totally suppressed.

Because of the difficult analytical quantification of the enantiomeric ratios of cysteine, insufficient attention has been paid in the past to the problem of cysteine racemization. In fact, the chiral analysis of hydrolysates of cysteine–peptides is complicated by the high and sequence-dependent rate of epimerization of cysteine over the time course of acid hydrolysis. This is due to thiazoline formation with the N-adjacent residue under acidic conditions and tautomerization of these intermediates [12]. Therefore, to assess quantitatively the percentage of hydrolysis-dependent racemization of cysteine the use of D_2O/

Address for correspondence: Prof. L. Moroder, Max-Planck-Institut für Biochemie, AG Bioorganische Chemie, Am Klopferspitz 18A, D-82152 Martinsried, Germany. Tel. 49-89-8578 3905; fax: 49-89-8578 2847.

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DCl for acid hydrolysis has been proposed [13]. By this procedure the total content of D-enantiomer can be determined by gas chromatography of the derivatized amino acid pool on Chirasil-Val glass capillary columns and the percentage of Cα-deuterated amino acids formed during hydrolysis by mass spectrometry via GC-MS coupling. Although the method allows the synthesis-dependent percentage of epimerization to be defined, it has not found the due widespread application since it requires experience and sophisticated equipment.

In the present communication we propose an alternative procedure that is based on the derivatization of the cysteine residues to acid-stable derivatives prior to acid hydrolysis in order to avoid thiazoline formation and thus to suppress the strong sequence-dependent rate of cysteine racemization in the hydrolysis step.

MATERIALS AND METHODS

Materials

4-Vinylpyridine and tris(2-carboxyethyl)phosphine were from Aldrich, Steinheim, Germany, tributylphosphine and 18-crown-6-tetracarboxylic acid from Fluka, Neu Ulm, Germany, and glutathione from Sigma, Deisenhofen, Germany; all reagents and solvents used were of the highest quality commercially available. The cystine- and cysteine-peptides analysed in this study were previously synthesized in our laboratory.

Derivatization of Cysteine-Peptides with 4-Vinylpyridine

Water-soluble cystine-peptides were reduced at 0.5 mM concentration in degassed and argon-saturated 10 mM TRIS–citrate buffer (pH 5.0) with a slight excess of tris(2-carboxyethyl)phosphine at 37 °C for 45 min [14]. Then 1 equiv. 4-vinylpyridine/SH was added and the reaction mixture was kept in the dark for 2 h to yield the *S*-β-(4-pyridylethyl)cysteine-peptides. The solution was adjusted to 6 M HCl with concentrated HCl and acid hydrolysis was performed in sealed evacuated ampoules at 110 °C for 24 h, if not stated otherwise, and for 4, 12, 24 and 48 h in the time-dependent experiments.

Water-insoluble cystine- or (*S*-*tert*-butylthio)-cysteine-peptides were reduced in 95% aqueous trifluoroethanol with a 10-fold excess of tributylphosphine for 12 h [15]. The solution was diluted with water and lyophilized. The residue was dissolved in 10 mM TRIS–citrate buffer (pH 7.0) or buffer/trifluoroethanol mixtures and reacted with 4-vinylpyridine. Acid hydrolysis of the derivatized samples was performed according to standard procedures.

Chiral Analysis of D,L-*S*-β-(4-Pyridylethyl)cysteine by Capillary Zone Electrophoresis

The acid hydrolysates were analysed on a Spectra Phoresis 1000 capillary electrophoresis apparatus (TSP, Darmstadt Germany) using an underivatized fused silica capillary (67 cm × 75 μm; length × ID). The electrophoretic separations were performed in 5 mM TRIS–citrate buffer (pH 2.3) with 20 mM 18-crown-6-tetracarboxylic acid at 25 °C with 15 kV after 1 s hydrodynamic injection. UV absorption at 250 nm was used to monitor the electropherogram; at this wavelength the non-aromatic amino acids are not detected. Analysis of amino acid standard mixtures has shown that under the conditions applied the natural aromatic amino acids exhibit higher t_R values and thus do not disturb detection of the *S*-β-(4-pyridylethyl)cysteine enantiomers.

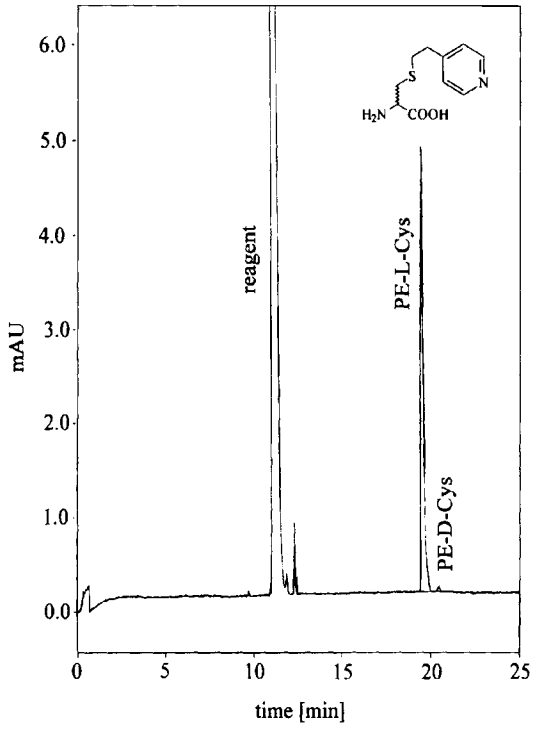

Figure 1 Enantiomeric resolution of D,L-*S*-β-(4-pyridylethyl)-cysteine by capillary zone electrophoresis in the presence of 18-crown-6-tetracarboxylic acid as chiral selector.

Chiral Analysis of D,L-S-β-(4-Pyridylethyl)cysteine by Gas Chromatography

The amino acid pools of the acid hydrolysates were derivatized to trifluoroacetyl-amino acid *n*-propyl esters according to Frank *et al.* [16]. The enantiomeric resolution of the cysteine derivative was performed on a Chirasil-Val glass capillary column using the gas chromatograph Fractovap (Carlo Erba, 4160).

RESULTS AND DISCUSSION

From protein chemistry it is well known that reaction of cysteine residues with 4-vinylpyridine proceeds quantitatively and with a high degree of selectivity, and that the resulting S-β-(4-pyridylethyl)cysteine is stable to acid hydrolysis [17]. This observation was fully confirmed in the present study; correspondingly, formation of thiazoline with concomitant racemization of both the N-adjacent amino acid residue and the cysteine is prevented. Enantiomeric resolution of amino acids, particularly of aromatic amino acids by capillary zone electrophoresis has successfully been achieved applying the host–guest complexation with crown ethers or cyclodextrins as chiral selectors [18–22]. The hydrophobic character of D,L-S-β-(4-pyridylethyl)cysteine was expected to allow for a similar efficient enantiomeric resolution. In fact, as shown in Figure 1, a base-line separation

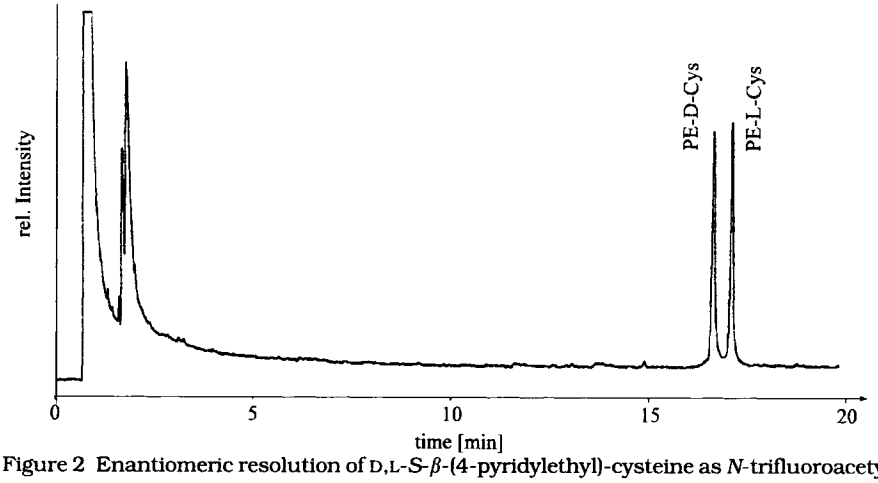

Figure 2 Enantiomeric resolution of D,L-S-β-(4-pyridylethyl)-cysteine as *N*-trifluoroacetyl and *n*-propyl ester derivative by gas chromatography on Chirasil-Val column.

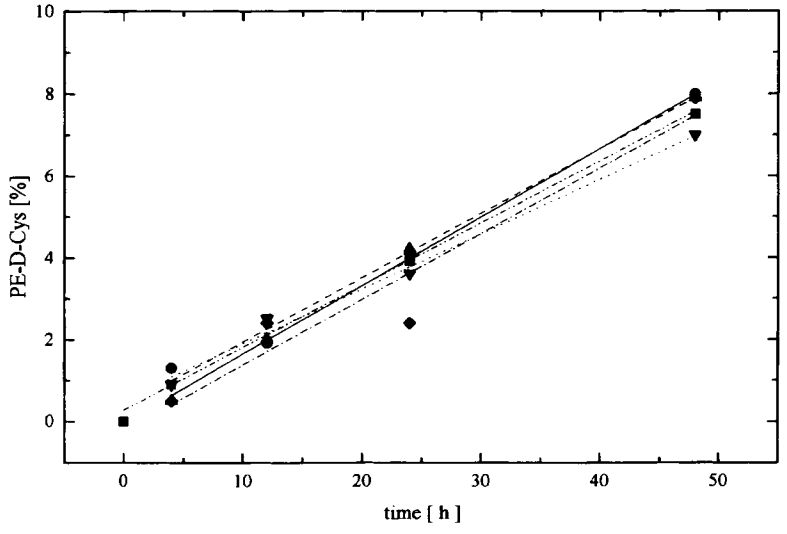

Figure 3 Time-dependent racemization of cysteine and related peptides upon S-4-pyridylethylation under conditions of acid hydrolysis: L-cysteine (■), glutathione (●), H-Cys-Pro-Val-OH (▲), H-Ala-Gly-Cys-OH (▼) and somatostatin (◆).

Table 1 D-Cysteine Contents in Acid Hydrolysates (6 M HCl; 24 h at 110 °C) of Synthetic Peptides after Derivatization of the Cysteine Residues with 4-Vinylpyridine Determined by Capillary Zone Electrophoresis

Sample	Reduction conditions	D-Cys (%)
L-Cys	–	3.9[a]
γ-Glu-Cys-Gly-OH	–	4.1[a]
H-Ala-Gly-Cys(StBu)-OH	A	3.6[b]
H-Cys(StBu)-Pro-Val-OH	A	4.2[b]
H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH	B	3.6[c]
Ac-Trp-Cys-Gly-Pro-Cys-Lys-His-Ile-NH_2	A	38.5[d]
Boc-Cys(StBu)-Tyr(tBu)-Ile-Gln-OH	A	9.4[b]
Z-Gly-Ile-Pro-Cys(StBu)-Cys(StBu)-Pro-Val-OH	A	4.8[b]
H-Lys(Boc)-Thr(tBu)-Phe-Thr(tBu)-Ser(tBu)-Cys(StBu)-OtBu	A	4.6[b]

Conversion to cysteine peptides was carried out by reduction with tributylphosphine (A) or tris(2-carboxyethyl)phosphine (B). D-Cysteine contents of *ca.* 4% correspond to the hydrolysis-dependent racemization of S-β-(4-pyridylethyl)cysteine (see Fig. 3).
[a] Commercial sample.
[b] Synthetic peptide intermediate obtained by conventional procedures in solution.
[c] Purified synthetic somatostatin.
[d] The high content of D-Cys was shown to derive from the TBTU/HOBt/DIEA coupling method used in the solid-phase peptide synthesis [8].

is achieved in the presence of 18-crown-6-tetracarboxylic acid as a chiral selector which permits quantification of D-cysteine contents of $\leq 1\%$. The enantiomeric resolution obtained by the gas chromatographic method upon suitable derivatization of D,L-S-β-(4-pyridylethyl)cysteine was found to be similarly efficient (Figure 2).

As expected from previous studies on amino acids [13], exposure of L-S-β-(4-pyridylethyl)cysteine to conditions of acid hydrolysis leads to a time-dependent degree of epimerization. However, the rate determined for this cysteine derivative was found to be significantly lower than that of underivatized cysteine and, more importantly, only marginally affected by the chemical environment, i.e. almost sequence-independent, as documented by the rates of racemization of the derivatized cysteine residues in peptides containing this residue in *endo*-positions or at the N- and C-terminals (Figure 3). This observation confirms our working assumption that prederivatization of cysteine prevents significant sequence-dependence in the acid-catalysed epimerization rate. Therefore, the determination of the hydrolysis-dependent degree of racemization under standard conditions is facilitated and a more precise quantification of D-cysteine contents deriving from synthetic procedures is obtained (see Table 1).

To conclude, this new method permits a fast racemization test for cysteine in synthetic peptides by capillary electrophoresis or gas chromatographic techniques. Moreover, it is conceivable that enantiomeric resolution of D,L-S-β-(4-pyridylethyl)cysteine can easily also be achieved by reversed-phase HPLC upon its derivatization with chiral isothiocyanates, e.g. with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate or with 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate [22, 23].

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