Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 838–844
Published online 11 February 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1012

JOURNAL OF PEPTIDE SCIENCE

A simple method for monitoring the cysteine content in synthetic peptides

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Received 31 October 2007; Revised 28 November 2007; Accepted 28 November 2007

Abstract: A new method for the determination of the cysteine content of synthetic peptides is described. For this purpose the classical amino acid analysis is not suitable, as cysteine may undergo oxidative dimerisation to cystine during the hydrolysis or evaporation. The intact peptide was reacted with *N*-ethylmaleimide (NEM) yielding a stable *S*-(*N*-ethylsuccinimido)-cysteine derivative, which could be separated by RP-HPLC and characterised by mass spectrometry. For the quantitative determination less than 0.1 μM peptide was sufficient. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide; cysteine; *N*-ethylmaleimide; HPLC; mass spectrometry

INTRODUCTION

Peptide/polypeptide and protein-based conjugates with covalently attached epitope peptides are considered as potential synthetic vaccine or diagnostics candidates and also for drug targeting to be used in chemotherapy [1]. Applications of cysteine-insertion and thioether linkage approaches to the preparation of a number of bioactive peptide conjugates are reported [1–3]. Peptides containing epitopes from (i) *Herpes simplex* virus type 1 glycoprotein D, (ii) a specific *N*-terminal β-amyloid epitope recognised by therapeutically active antibodies, (iii) GnRH-III peptide from sea lamprey with anti-tumour activity, (iv) *Mycobacterium tuberculosis* related epitope peptides, (v) cytokines and their receptors related peptides were elongated with Cys residues and attached to chloroacetylated carrier derivatives via a thioether linkage either directly or by insertion of a spacer [2,3]. An advantage of such a direct linkage is the non-antigenic character of the linkage compared with some of those introduced with hetero-bifunctional coupling agents [2,4]. The structure and molecular homogeneity of all the peptide conjugates were ascertained by HPLC, MALDI and electrospray mass spectrometry. Experimental data showed that the purity of the Cys elongated peptides and the attachment site of the Cys residues to the epitope peptides (*N*- or *C*-terminal elongation), have significant effects on the quality of the final conjugates and the yield of the coupling reactions.

In controlling the purity of synthetic peptides, quantitative amino acid analysis is still one of the most important methods. After hydrolysis the building amino acids can be separated by chromatography and determined using pre-column or post-column derivatisation. In the case of cysteine-containing peptides one has to take into account some difficulties. It is well known that cysteine is very sensitive to oxidation. Even under carefully selected reaction conditions cysteine residues can be easily oxidised to cystine. A similar side-reaction can occur during synthesis as well, it is not easy to decide whether the cystine determined by the amino acid analysis originated from the hydrolysate or the investigated peptide was contaminated. On ion-exchange chromatography the retention time of proline and cysteine is almost the same, therefore cysteine can be determined only in the case of a proline free peptide. Choosing the other well-known method, the pre-column derivatisation by ortho-phthaldialdehyde (OPA), another difficulty should be taken into consideration. To stabilise the isoindole derivative originating during the reaction, one has to use a sulfhydryl-containing compound. It is clear that in such circumstances the determination of cysteine is theoretically almost impossible. To solve these problems, in the early period of amino acid analysis a procedure was proposed [5] in which the cysteine of a protein or peptide was oxidised and determined as cysteic acid. This method has some disadvantages. Amino acids sensitive to oxidation, e.g. methionine, may also react and cysteic acid may originate from cystine impurities.

For the determination of free sulfhydryl-groups in proteins, Ellman's method is commonly used [6]. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) is a symmetrical aryl disulfide that readily undergoes a thiol–disulfide interchange reaction in the presence of free sulfhydryl-groups forming the corresponding thionitrobenzoic acid (TNB), which can be determined spectrophotometrically. The reaction has been successfully applied for different proteins, especially in measuring the activity of acetylcholinesterase [7,8]. In spite of its widespread popularity the method has some practical problems, e.g. pH-induced disulfide disruption and

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the re-oxidation of the R-SH groups [9]. Though the reaction is quantitative, the fact that the product to be measured is the result of a second reaction step can cause some difficulties. Among the reaction parameters neutral pH, temperature and the oxygen-free medium are very important. Even when these conditions are carefully controlled anomalies may be observed [10,11].

The reaction of a free sulfhydryl-group with *N*-ethylmaleimide (NEM) is well known. The formation of the adduct has been followed by spectrophotometric measurement using the decrease of the absorbance at $\lambda = 300$ nm [12–14]. The cysteine content of several proteins has been determined using this method. The stability of NEM in solution and the rate of the reaction between NEM and glutathione at different pH values have also been investigated [15]. Many attempts have been made to improve the method, in order to make the determination of the sulfhydryl–maleimide adduct easier. For example, the cysteine content of different proteins was determined using a coloured maleimide-derivative [16], fluorescent maleimide-derivative [17] and various *N*-substituted maleimides [18]. In the case of polypeptides or proteins of low molecular weight, the number of free sulfhydryl-groups can be determined by the reaction with 4-vinylpyridine or NEM followed by mass spectrometry [11]. A series of *N*-polymethylenecarboxymaleimides has been synthesised as membrane-permeant sulfhydryl-reagents and by this means the position of sulfhydryl-groups within the membrane has been investigated [19]. Studying the redox-status of cysteine, homocysteine and glutathione in human plasma, NEM was used for blocking the free sulfhydryl-groups [20].

Our aim was to develop a new method based on an unequivocal reaction course leading to a stable reaction-product easy to measure for determination of free sulfhydryl-group of synthetic, cysteine containing peptides. For this purpose the reaction between the free sulfhydryl-group and NEM seemed to be a suitable one. By this new method it is possible to determine the cysteine content in the intact peptide. The reaction with NEM is very simple (Figure 1), in neutral or slightly basic medium it takes place in a few minutes. The product, the *S*-(*N*-ethylsuccinimido)-cysteinyl-peptide can be measured by RP-HPLC, the starting substance and the unreacted reagent (NEM) could be separated efficiently and the product was confirmed by mass spectrometry. The relative amount of reduced and oxidised Cys-peptide was calculated from the peak area. In this article, we restrict ourselves to present only a few, very characteristic, examples of determination of SH-groups by this method developed in our laboratory. As reference substances *N*-acetylcysteine and glutathione have been used.

MATERIALS AND METHODS

Materials

NEM and L-glutathione were purchased from Fluka (Buchs, Switzerland). *N*-Acetylcysteine was obtained from Sigma (Steinheim, Germany), all other amino acid derivatives from

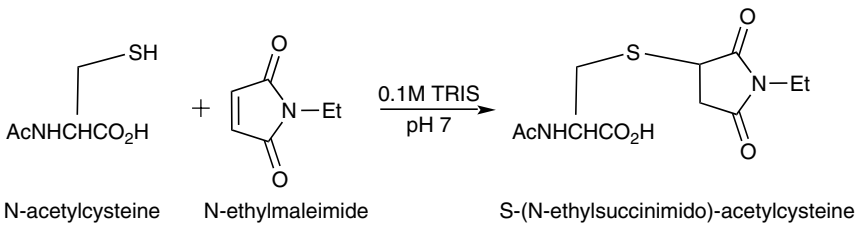

Figure 1 Reaction of *N*-ethylmaleimide with *N*-acetylcysteine.

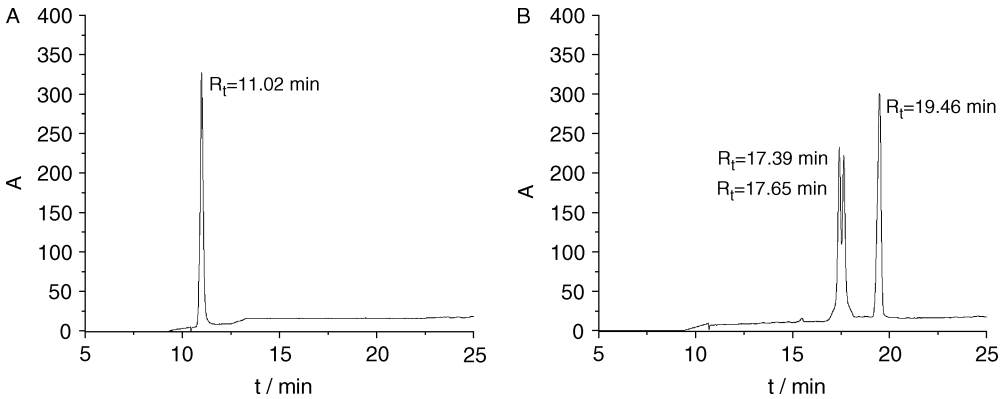

Figure 2 RP-HPLC analysis of *N*-acetylcysteine dissolved in 0.1M TRIS buffer (pH = 7.0, $c = 1$ mg/ml) (A) and of the reaction mixture with NEM (1.1 M equivalent) (B). The eluent B content was from 0 to 40% in 20 min.

DOI: 10.1002/psc

Reanal (Budapest, Hungary). Acetonitrile and trifluoroacetic acid (both HPLC grade) were from Merck (Darmstadt, Germany).

Peptide Synthesis and Purification

The sequences of the analysed peptides are as the following: SEFAYGSFVRTVSLPVC, DQVHFQPLPPAAVVC, IRVRNASWQHDIDSLFCTQR (peptides derived from immunodominant proteins of *M. tuberculosis*), SEWSC (highly conserved interleukin-6 receptor domain). All peptides were synthesised on solid phase using Fmoc/*t*Bu chemistry as described in detail elsewhere [3,21,22]. After cleavage from the resin the peptides were purified by RP-HPLC on semi-preparative C-18 column. Peptides were of minimum 90% purity and homogeneity was confirmed by analytical RP-HPLC, mass spectrometry and amino acid analysis.

Reaction of Free Thiol Group with N-Ethylmaleimide

All peptides and amino acid derivatives were dissolved at the concentration of 1 mg/ml in 0.1M TRIS buffer (pH 7.0). NEM was dissolved in absolute ethanol to obtain a stock solution ($c = 0.1$ mg/ml), freshly prepared daily. To the peptide solution, 1.1 M equiv of NEM to 1 equiv free thiol group was added. The mixture was sonicated at room temperature for 5 min. There were no significant differences in peak areas of the succinimide derivatives when samples were incubated at room temperature from 5 to 60 min.

Amino Acid Analysis

Amino acid analysis was performed on a Sykam Amino Acid S433H analyser (Eresing, Germany).

High-Performance Liquid Chromatography

Peptides, amino acid derivatives and the reaction mixtures were analysed by RP-HPLC using a laboratory-assembled Knauer HPLC system (Bad Homburg, Germany) with an Eurospher-100 C-18 column (250 mm × 4 mm I.D., 5 µm particle size, 300 Å pore size) (Knauer, Bad Homburg, Germany). The gradient elution system consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water = 80/20 (v/v) (eluent B). The eluent B content varied depending on the polarity of the analysed product. The flow rate was 1ml/min at room temperature. 20 µl of samples were injected and peaks were detected at $\lambda = 214$ nm.

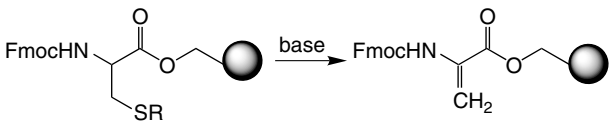

Figure 4 Formation of dehydroalanine by β-elimination of *C*-terminal cysteine residue during solid phase synthesis.

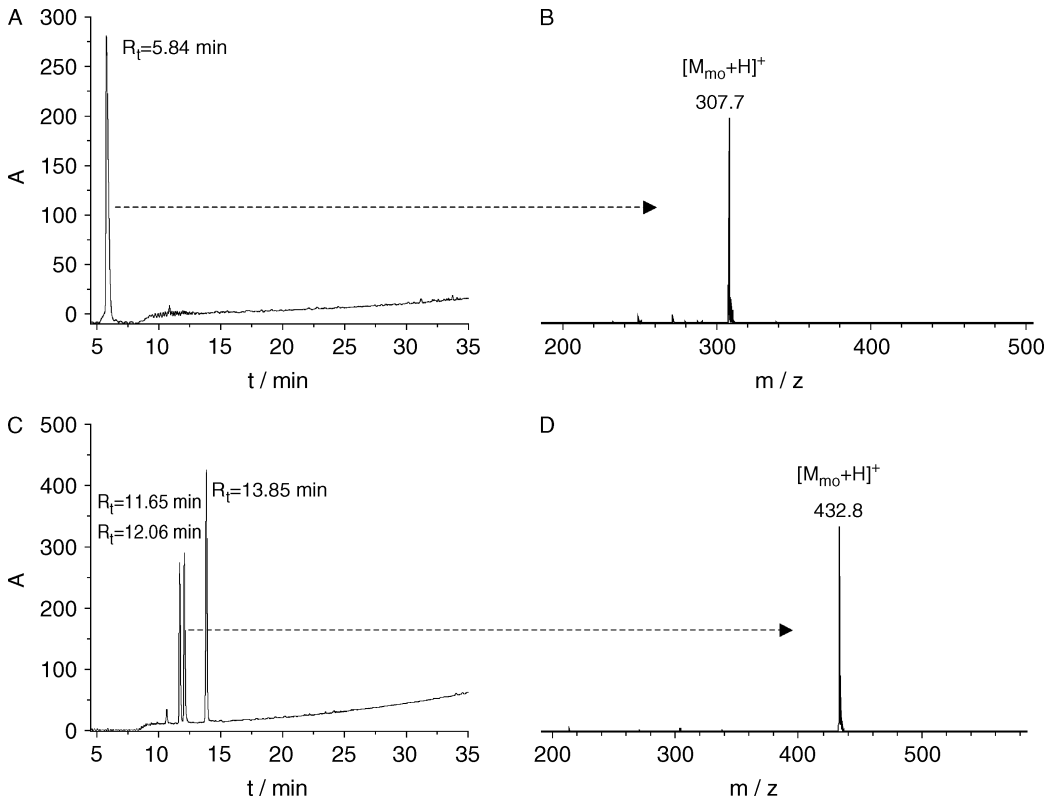

Figure 3 Analytical RP-HPLC chromatogram (A) and ESI mass spectra (B) of glutathione. After adding 1.1 M excess of NEM ($R_t = 13.85$ min) the product eluated in two peaks ($R_t = 11.65$ min and $R_t = 12.06$ min) (C). The eluent B content was from 2 to 60% in 30 min. The molecular mass of the target compound was confirmed by ESI MS (D).

DOI: 10.1002/psc

Mass Spectrometry

Mass spectrometric analyses were performed on a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker, Bremen, Germany) equipped with electrospray ionisation (ESI) source. Spray voltage was set to 4.0 kV, and 40.0 V orifice voltage was applied. Samples were dissolved in a mixture of acetonitrile/water = 1/1 (v/v) containing 0.1% acetic acid and introduced by a syringe pump with a flow rate of 10 µl/min. The instrument was used in positive mode in the range of 50–3000 m/z with 13 000 m/z/sec scan resolution.

Time Dependence of Dimerisation of SEFAYGSFVRTVSLPVC Peptide

To monitor the rate of dimerisation, the peptide was dissolved in 0.1M TRIS (pH 8.3), (c = 1 mg/ml) and stirred at room temperature. In every hour, to 100 µl (100 µg, 53.8 µmol)

Table 1 Analytical characterisation of peptides and peptide derivatives

Peptide	M_{mo}[a]	R_t[b] (min)	M_{mo} of succinimido derivative[a]	R_t of succinimido derivative[b] (min)
glutathione	306.7	5.84	431.8	11.65/12.06
SEWSC	609.9	20.52	734.9	23.78
SEFAYGSFVRTVSLPVC	1860.1	32.83	1958.2	34.25
DQVHFQPLPPAAVVC	1450.2	28.62	1575.2	30.31
IRVRNASWQHDIDSLFCTQ	2444.4	31.10	2569.6	32.31

[a] Monoisotopic molecular mass measured by ESI MS.
[b] Retention time on analytical RP-HPLC chromatogram.

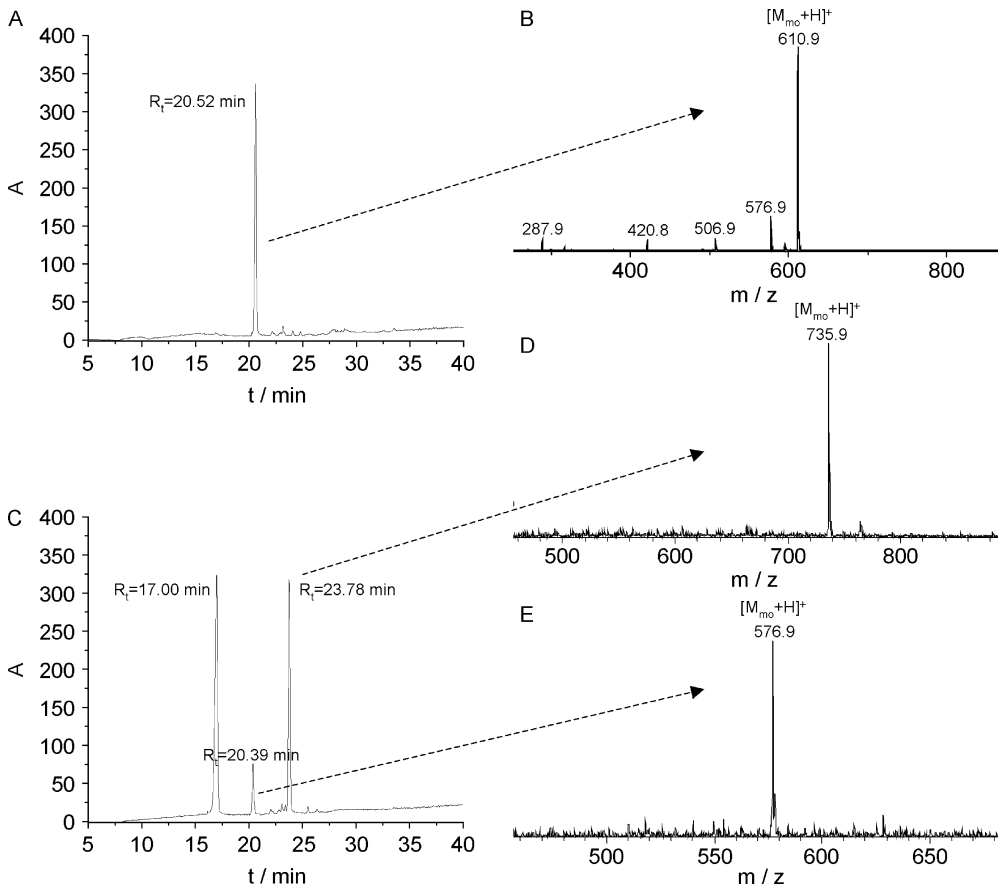

Figure 5 Analytical RP-HPLC chromatogram (A) and ESI mass spectrum (B) of SEWSC peptide. Chromatogram C shows the reaction of the peptide with NEM (R_t = 17.00 min), the resulting succinimido derivative (R_t = 23.78 min) and the byproduct containing dehydroalanine (R_t = 20.39 min). Molecular mass values of these compounds are shown in spectrograms D and E, the latter being by 34.0 Da lower than that of the intact peptide (indicating the formation of dehydroalanine derivative).

DOI: 10.1002/psc

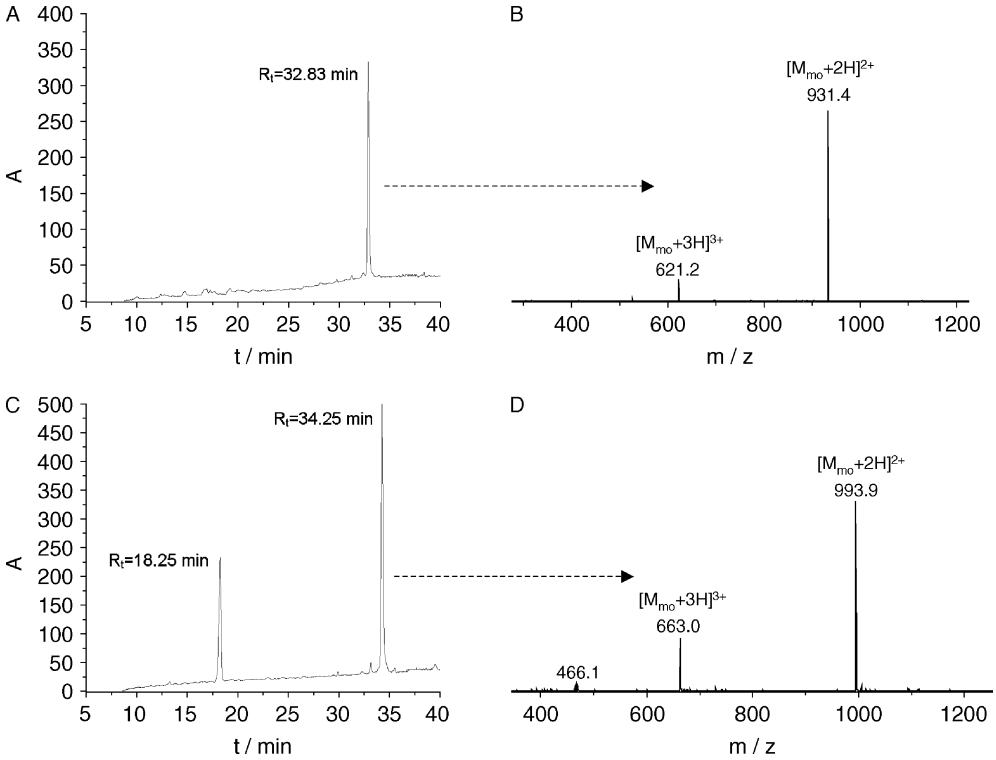

Figure 6 Analysis of SEFAYGSFVRTVSLPVC peptide by analytical RP-HPLC (A) and ESI MS (B). The eluent B content was from 2 to 60% in 35 min. The reaction with NEM (R_t = 18.25 min) resulted in the succinimide derivative (R_t = 34.25 min) (C), the structure of which was confirmed by ESI MS (D).

sample 1.1 M equivalent (7.4 μg, 59.1 μmol) NEM was added and the reaction mixture was analysed by RP-HPLC.

RESULTS

The reaction between cysteine and NEM leads to a compound *S*-(*N*-ethylsuccinimido)-cysteine. (Figure 1). As expected the resulting product contains the equimolar mixture of the S,R and S,S diastereomers, which can be separated by analytical RP-HPLC in the case of smaller molecules.

The HPLC chromatograms presented in Figure 2 clearly shows the disappearance of the starting material (R_t = 11.02 min) and the appearance of two diastereomers (R_t = 17.39 and 17.65 min), as well as the presence of the excess reagent (R_t = 19.46 min).

In the reaction of the glutathione with NEM the diastereomers of the product appear again. The structure of the product from the isolated fraction of the HPLC run was confirmed by mass spectrometry (Figure 3).

The diastereomers obtained from synthetic peptides of higher molecular mass during the reaction with NEM were not demonstrable, perhaps due to the minimal differences in their retention times under the conditions used.

An unexpected reaction has been encountered in the course of determination of the sulfhydryl-group in the SEWSC peptide. The peptide on HPLC chromatogram seemed to be homogeneous, but after the reaction with NEM beside the reaction-product and the excess NEM a new peak appeared. At the investigation of this fraction by mass spectrometry the new peak proved to be a dehydroalanine containing contamination (Figure 5), which could have originated from cysteine by β-elimination during the synthetic steps (Figure 4).

Two of the investigated peptides are worth mentioning, both from the sequence of the immundominant protein of the *M. tuberculosis*, namely, a heptadecapeptide amide (SEFAYGSFVRTVSLPVC) and a tridecapeptide amide (DQVHFQPLPAVVC). By the amino acid analysis – because of the presence of proline – the determination of the cysteine residue is impossible, their cysteine content could be demonstrated only by mass spectrometry. The reaction with NEM supported the correct structure of the peptides in both cases. At the heptadecapeptide using the reaction with NEM, even the time dependence of dimerisation of the peptide could be followed. The decrease of the amount of free sulfhydryl-containing peptide was measured for 8 h, when the conversion was about 80% and the reaction became very slow. Figure 6 shows the reaction of the heptadecapeptide with NEM.

In the Table 1 we summarise our results, showing the molecular mass and retention time values of starting peptides and the product with NEM.

DOI: 10.1002/psc

DISCUSSION

The purity of the synthetic peptides was controlled in every case by amino acid analysis. Though the peptides were homogeneous by HPLC and their structure was supported by mass spectrometry, we could not detect cysteine in the hydrolysate in the calculated amount. In those cases, when the sequence did not contain proline whose presence could disturb the determination of cysteine by amino acid analysis, the cysteine content appeared mostly as cystine. It is very likely that the milieu of the hydrolysis and the succeeding evaporation are responsible for the formation of cystine, but even under carefully controlled reaction conditions a partial oxidation of cysteine could not be avoided. In the case of amino acid analysis of glutathione, as a reference substance, the cysteine–cystine ratio was 85 : 15.

According to the literature, [23,24] the NEM-derivative of cysteine-containing peptides can be hydrolysed and determined by amino acid analysis. The product with NEM is *S*-(*N*-ethylsuccinimido)-cysteine, which yields equal amounts of cysteine-*S*-succinic acid and ethylamine during the hydrolysis. At ion-exchange chromatography, the expected cysteine derivative elutes before aspartic acid and ethylamine after the basic amino acids. However, in the case of glutathione, not only the succinic acid derivative but also free cysteine was detected after the hydrolysis. This amino acid could be originated only from the partial decomposition of the thioether compound. It is certainly possible to eliminate this side-reaction by modifying the hydrolysis parameters; however, we are convinced that for the determination of the cysteine content in the intact peptides, the reaction with NEM and the monitoring by HPLC, the resulting *S*-(*N*-ethylsuccinimido) compound is the most promising method as described in this article. A great advantage of it is to provide a selective, chemically defined and safe way to tell apart dimer from the monomer originally containing free sulfhydryl-group. As it was shown, the method is suitable to detect side-reactions as well. The β-elimination step is possible in the case of cysteine-containing peptides and it is sometimes hard to determine peptides of higher molecular mass by HPLC. Furthermore, it is easy to follow a dimerisation reaction between two cysteine residues by this procedure. For the determination of the sulfhydryl content by this procedure, less than 0.1 μM of the synthetic peptide is sufficient.

Acknowledgement

The authors are grateful to Hungarian Research Fund (T49814 and GVOP 3.2.1-2004-04-0352/3.0) for financial support.

REFERENCES

1. Hudecz F. Synthesis of peptide bioconjugates. *Methods Mol. Biol.* 2005; **298**: 209–223.
2. Mezo G, Manea M, Jakab A, Kapuvári B, Bosze S, Schlosser G, Przybylski M, Hudecz F. Synthesis and structural characterization of bioactive peptide conjugates using thioether linkage approaches. *J. Pept. Sci.* 2004; **10**(12): 701–703.
3. Bosze SZ, Mezo G, Caccamo N, Dieli F, Andreu D, Tsikaris V, Sakarellos V, Hudecz F. Synthesis and in vitro effect of peptide-conjugates from 38 kDa protein of *M.tuberculosis* on T-cell proliferation and IFN-γ production. In *Peptides 2004*, Flegel M, Fridkin M, Gilon C, Slaninova J (eds). Kenes International: Israel, 2005; 1105–1106.
4. Boeckler C, Frisch B, Muller S, Schuber F. Immunogenicity of new heterobifunctional cross-linking reagents used int he conjugation of synthetic peptides to liposomes. *J. Immunol. Methods* 1996; **191**: 1–10.
5. Schram E, Moore S, Bigwood EJ. Chromatographic determination of cysteine as cysteic acid. *Biochem. J.* 1954; **57**: 33–37.
6. Ellman GL. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 1959; **82**: 70–77.
7. Ellman GL, Gourtney D, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 1961; **7**: 88–90.
8. Garry PJ, Routh JI. A micro method for serum cholinesterase. *Clin. Chem.* 1965; **11**: 91–96.
9. Riddles PW, Blakeley RL, Zerner B. Reassessment of Ellman's reagent. *Meth. Enzymol.* 1983; **91**: 49–60.
10. Gergel D, Cederbaum AI. Interaction of nitric oxide with 2-Thio-5-nitrobenzoic acid: Implication for the determination of free sulfhydryl Groups by Ellman's reagent. *Arch. Biochem. Biophys.* 1997; **347**: 282–288.
11. Ming D, Markley JL, Hellekant G, Gergel D, Cederbaum AI. Quantification of Cysteinyl sulfhydryl residues in peptides and proteins by ESI-MS or Maldi-MS. *BioTechniques* 1995; **18**(5): 808.
12. Friedmann E. Spectrophotometric investigations of the interaction of glutathione with maleimide and N-ethylmaleimide. *Biochim. Biophys. Acta* 1952; **9**: 65–75.
13. Alexander NM. Spectrophotometric assay for Sulfhydryl Groups using N-ethylmaleimide. *Anal. Chem.* 1958; **30**: 1292–1294.
14. Roberts E, Rouser G. Spectrophotometric assay for reaction of N-Ethylmaleimide with sulfhydryl groups. *Anal. Chem.* 1958; **30**: 1291–1292.
15. Gregory JD. The stability of N-Ethylmaleimide and its reaction with sulfhydryl groups. *J. Am. Chem. Soc.* 1955; **77**: 3922–3923.
16. Witter A, Tuppy H. N-(4-Dimethylamino)-3,5-Dinitrophenyl) maleimide: A coloured sulfhydryl reagent: Isolation and investigation of cysteine-containing peptides from human and bovine serum albumin. *Biochim. Biophys. Acta* 1960; **45**: 429–442.
17. Kanaoka Y, Machida M, Machida M, Sekine T. Fluorescent thiol reagents: VI: N-(1-Anilinonaphtyl-4)maleimide; a fluorescent hydrophobic probe directed to thiol groups in protein. *Biochim.Biophys.Acta Protein Structure* 1973; **317**: 563–568.
18. Arndt DJ, Simon SR, Maita T, Konigsberg W. The Characterization of chemically modified hemoglobins. III. Reaction with various N-substituted maleimides. *J. Biol. Chem.* 1971; **246**: 2602–2608.
19. Griffiths DG, Partis MD, Sharp RN, Beechey RB. N-polymethylene-carboxymaleimides–a new class of probes for membrane sulfhydryl groups. *FEBS Lett.* 1981; **134**: 261–263.
20. Mansoor MA, Svardal AM, Ueland PM. Determination of the in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Anal. Biochem.* 1992; **200**: 218–229.
21. Bosze SZ, Caccamo N, Majer Z, Mezo G, Dieli F, Hudecz F. In vitro T-cell immunogenicity of oligopeptides derived from the region 92–110 of the 16 kDa protein of *Mycobacterium tuberculosis*. *Biopolymers* 2004; **76**(6): 467–476.

22. Biró J, Bosze Sz, Hudecz F, Nagy Z, Rajnavölgyi E, Schmidt B, Rákász E, Falus A. The effect of WSEWS pentapeptide and WSEWS-specific monoclonal antibodies on constitutive and IL-6 induced acute-phase protein production by a human heptoma cell line, HEPG-2. *Immunol. Lett.* 1995; **46**: 183–187.
23. Guidotti G, Konigsberg W. The characterization of modified human hemoglobin. I. reaction with Iodoacetamide and N-ethylmaleimide. *J. Biol. Chem.* 1964; **239**: 1474–1484.
24. Spackman DH, Stein WH, Moore S. Automatic recording apparatus for use in chromatography of amino acids. *Anal. Chem.* 1958; **30**: 1190–1206.

DOI: 10.1002/psc