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Determination of glyoxylyl-peptide concentration using oxime chemistry and RP-HPLC analysis

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Abstract: Glyoxylyl-peptides are useful peptide derivatives in the context of hydrazone, oxime or thiazolidine ligations. We describe a method for the determination of glyoxylyl-peptide concentration based on the reaction of the α-oxo aldehyde group with an excess of *O*-benzylhydroxylamine. The amount of *O*-benzylhydroxylamine necessary to convert the α-oxo aldehyde group into the corresponding *O*-benzyloxime was determined by RP-HPLC analysis and corresponded to the quantity of glyoxylyl-peptide used in the experiment. The method is rapid, sensitive, accurate and allows the automated analysis of several samples. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: glyoxylyl-peptide; oxime; *O*-benzylhydroxylamine

INTRODUCTION

Peptide chemists have developed site-specific ligation methods that allow the assembly of unprotected peptide fragments into larger structures in an aqueous solution. The α-oxo aldehyde group plays a key role in this field due to its stability, its ease of incorporation using solution or solid-phase methods [1–9] and its usefulness for oxime [10–12], hydrazone [13] or thiazolidine [14] bond formation. α-Oxo aldehyde chemistry also has interesting applications in the preparation of hybrid bioorganic–inorganic materials or of peptide microarrays [15,16].

For all these applications, it is important to know the exact concentration of glyoxylyl-peptide used in the ligation experiment. The reference method for the quantification of glyoxylyl groups in peptides or proteins is based on the reaction of the carbonyl compound with an excess of 2,4-dinitrophenylhydrazine (DNPH) in aqueous HCl followed by detection of the resulting hydrazone by UV spectroscopy [17,18]. However, the accurate determination of aldehyde concentration requires a standard curve for each aldehyde and the UV-monitoring of the reaction for 1 h.

We present here an alternative method for the determination of glyoxylyl-peptide concentration based on oxime chemistry and RP-HPLC analysis. A standard curve is not required and the analysis of several samples can be automated using an HPLC machine equipped with an automatic injector.

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MATERIAL AND METHODS

Peptide Synthesis

Peptide 2a. Peptide **2a** (0.1 mmole scale) was synthesised on a 4,7,10-trioxa-1,13-tridecanediamine functionalised isopropylidene tartrate NovaSyn® TG resin (loading 0.17 mmol/g) using the Fmoc/*tert*-butyl strategy [19] on a PerSeptive Pioneer peptide synthesiser. The preparation of the solid support is described in detail in Ref. 6. Fmoc-protected alanine (single couplings, 10 eq) was purchased from Novabiochem. The amino acids were activated using TBTU/HOBt (0.5 M) and DIEA (1 M) in DMF. The peptidyl-resin was capped with Ac_2O/DIEA/DMF : 3/0.3/96.7 by vol. following each coupling. After peptide elongation, the resin was washed with CH_2Cl_2 and dried *in vacuo*. On-resin deprotection was performed with TFA/TIS/H_2O : 9.5 ml/0.25 ml/0.25 ml during 2 h at r.t. The resin was washed with TFA (2 × 2 min), CH_2Cl_2 (2 × 2 min), MeOH (2 × 2 min) and water/AcOH : 2/1 by vol. (2 × 5 min).

Cleavage from the solid support was performed by adding 128.3 mg (0.600 mmol) of $NaIO_4$ to the resin suspended in 7 ml of water/AcOH : 2/1 by vol. The resin was agitated for 2 min 30 s and then the excess of periodate was quenched with 145 µl (2.40 mmol) of ethanolamine. The resin was filtered, washed twice with 6 ml of water and the combined filtrates were injected on a C3 Zorbax column. Eluent A: deionised water containing 0.05% TFA, eluent B: acetonitrile/deionised water: 4/1 by vol. containing 0.05% TFA, 0–40% B in 30 min, 3 ml/min. 750 mg of mannitol were added to the pure fractions before lyophilization. COCHO : 19.6 nmol/mg, yield 38%, MALDI-ToF $[M + H]^+$ m/z calcd 588.9, found 589.3.

Peptide 2b. The seryl precursor of peptide **2b** H-SILKEPVHGV-NH_2 was synthesised on a 0.2 mmol scale on a NovaSyn® TGR resin (0.29 mmol/g, Novabiochem) using standard Fmoc/*tert*-butyl strategy [19] on a PerSeptive Pioneer peptide synthesiser. Purification was performed on a C18 Hyperprep column (15 × 300 mm) using the above eluents (0–100% B in 90 min, flow rate 3 ml/min, detection at 230 nm) to give 118 mg (41% yield) of peptide H-SILKEPVHGV-NH_2.

100 mg (0.07 mmol) of seryl precursor was reacted for 10 min with 30.0 mg (0.14 mmol) of sodium periodate in 20 ml of 0.1 M pH 7.0 sodium phosphate buffer. The reaction was stopped by adding 34 µl (0.56 mmol) of ethanolamine. The crude reaction mixture was purified immediately by RP-HPLC on a C18 Hyperprep column (15 × 300 mm) as described for the seryl precursor to give 82 mg of peptide **2b** (91% yield, overall yield 37%). MALDI-TOF: $[M + H]^+$ m/z calcd 1046.59, found 1046.56.

Typical Experimental Procedure for the Determination of Glyoxylyl-Peptide Concentration. The validation of the titration method was performed according to published guidelines [20]. Samples were injected on a C4 Modulo-cart QS Uptisphere 5 Interchrom (4.6 × 250 mm) column using the above eluents (0–100% B in 30 min, 1 ml/min, 50 °C, detection at 215 nm).

20 µl of *O*-benzylhydroxylamine dissolved in 0.1 M pH 4.75 sodium acetate buffer at various concentrations were analysed as described above. The relationship between the area of the *O*-benzylhydroxylamine peak and its concentration was found to be linear up to 1.5 mM, thus permitting the direct analysis of the reaction mixture without the risk of saturating the HPLC system used in this study.

23.9 mg (0.150 mmol) of *O*-benzylhydroxylamine hydrochloride (99%, Acros Chemicals) were dissolved in 100 ml of 0.1 M pH 4.75 sodium acetate buffer (final concentration 1.50 mM, C_0). 20 µl of this solution was analysed by RP-HPLC and the peak area for *O*-benzylhydroxylamine was determined (A_0).

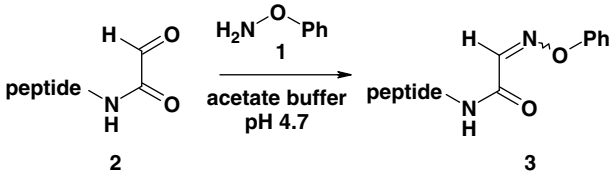
100 µl (150 nmol) of the *O*-benzylhydroxylamine solution was added to the glyoxylyl derivative (concentration C_{ald}). The mixture was heated for 4 h at 37 °C, analysed by RP-HPLC and the peak area for *O*-benzylhydroxylamine was determined (A). The concentration of glyoxylyl-peptide is given by $(1 - A/A_0) \times C_0$.

Application to peptide 2b. 3.87 mg of peptide **2b** was dissolved in 1 ml of water. 30 µl of this solution was transferred into polypropylene vials and dried at r.t. *in vacuo*. The peptide was analysed as described above. The glyoxylyl-peptide concentration found was 0.58 mM. The peptide concentration determined by quantitative amino acid analysis after acid hydrolysis was 0.56 mM.

RESULTS AND DISCUSSION

Principle of the Method

The principle of the method is described in Figure 1. An excess of *O*-benzylhydroxylamine (concentration C_0) is reacted with the glyoxylyl-peptide (concentration C_{ald}), which is converted quantitatively into the corresponding *O*-benzyloxime using appropriate experimental conditions (Scheme 1). The reaction mixture and the blank without glyoxylyl-peptide are analysed by RP-HPLC and the peak area for *O*-benzylhydroxylamine is determined (A and A_0 for the reaction and the blank respectively). The concentration of glyoxylyl-peptide C_{ald}, which can be deduced from the quantity of *O*-benzylhydroxylamine consumed in the reaction, is given by $(1 - A/A_0)C_0$. Using this method, the analysis of n samples requires $n + 1$ RP-HPLC analyses.



Scheme 1 Synthesis of *O*-benzyloxime **3** by reaction of glyoxylyl-peptide **2** with *O*-benzylhydroxylamine **1**.

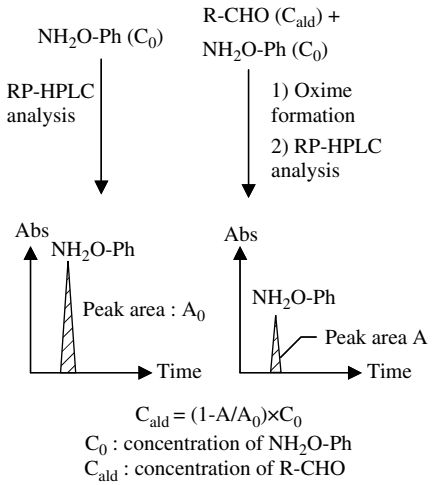


Figure 1 Determination of glyoxylyl-peptide concentration (C_{ald}) by reaction with *O*-benzylhydroxylamine (C_0) and RP-HPLC analysis. A_0 and A correspond to the peak areas of *O*-benzylhydroxylamine without (blank) and with glyoxylyl-peptide respectively.

Optimisation of O-Benzyloxime Formation

Model peptide Ac-AAAG-$NH(CH_2)_3(OCH_2CH_2)_2O(CH_2)_3$NHCOCHO **2a** (Figure 2), synthesised as described in detail elsewhere [6] using an isopropylidenetartrate-based linker and the Fmoc/*tert*-butyl chemistry, was used to optimise *O*-benzyloxime formation. Two equivalents of *O*-benzylhydroxylamine were reacted with peptide **2a** in a 0.1 M pH 4.7 sodium acetate buffer. Figure 3(A) shows the ratio between the peak area of *O*-benzylhydroxylamine with and without glyoxylyl-peptide **2a**. Heating the reaction mixture from 23 °C to 37 °C or increasing the concentration of *O*-benzylhydroxylamine accelerated the formation of *O*-benzyloxime. At 0.15 mM, the equilibrium was not attained after 4 h at 37 °C. Alternately, the equilibrium was reached in less than 3 h at 1.5 mM and about one half of the *O*-benzylhydroxylamine **1** was consumed in the reaction as expected (molar ratio **1**/**2a** : 2). All the starting glyoxylyl-peptide **2a** was converted into the corresponding *O*-benzyloxime **3a** as shown in Figures 3(B) and 4.

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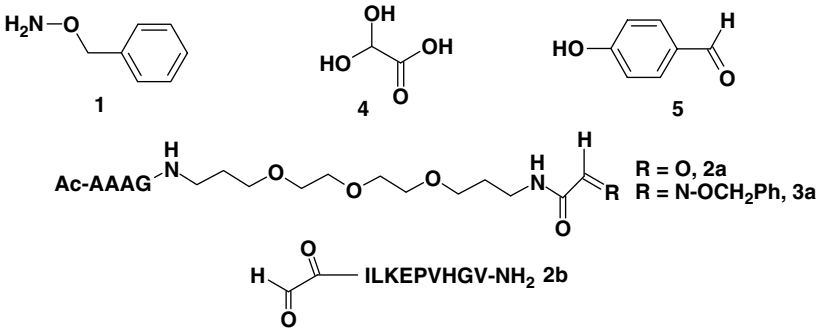

Figure 2 Compounds used in this study.

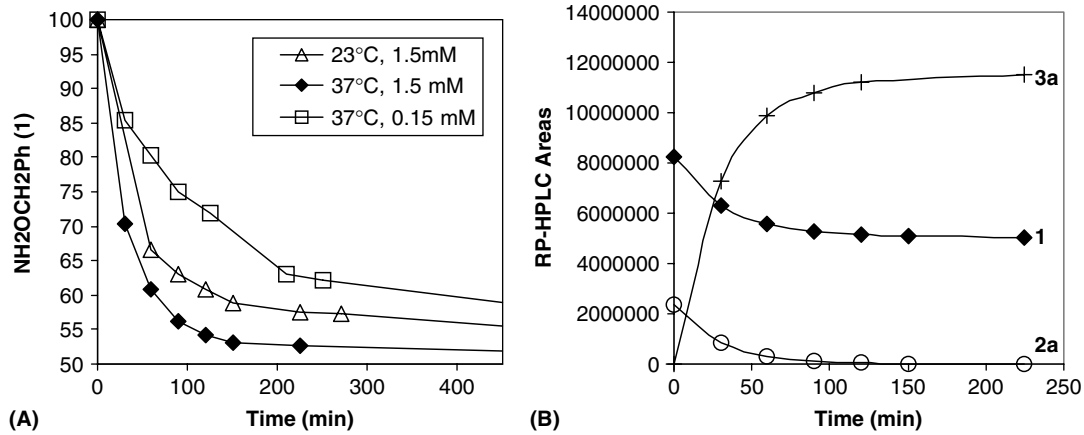

Figure 3 Effect of temperature and concentration of *O*-benzylhydroxylamine **1** on *O*-benzyloxime **3a** formation (molar ratio **1**/**2a** : 2). (A) Ratio between the peak areas of *O*-benzylhydroxylamine (1.5 or 0.15 mM) with and without glyoxylyl-peptide **2a**. (B) Reaction of **1** (1.5 mM) with peptide **2a** at 37 °C. Peak areas of *O*-benzylhydroxylamine **1**, glyoxylyl-peptide **2a** and *O*-benzyloxime **3a** are in arbitrary units.

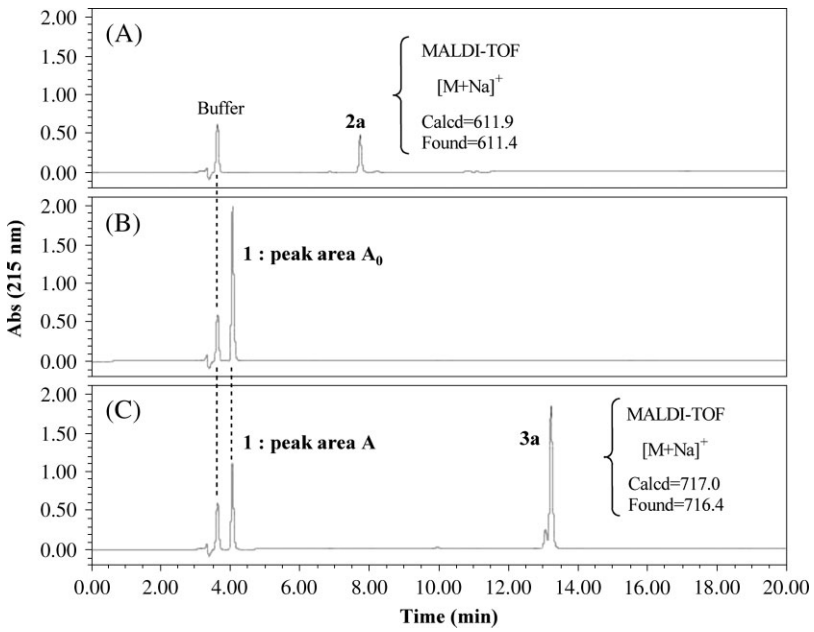

Figure 4 RP-HPLC traces of (A) peptide **2a**, (B) *O*-benzylhydroxylamine 1.5 mM in acetate buffer, determination of peak area A_0, (C) Crude reaction mixture at 37 °C after 4 h, determination of peak area *A*. Detection at 215 nm, 50 °C, C4 Modulo-cart QS Uptisphere 5 Interchrom (4.6 × 250 mm) column, 1 ml/min, eluent A: deionised water containing 0.05% TFA, eluent B: acetonitrile/deionised water: 4/1 by vol. containing 0.05% TFA, 0–100% B in 30 min.

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Validation of the Method

In a first approach, glyoxylic acid monohydrate **4** and p-hydroxybenzaldehyde **5** (Figure 2) were used to show that (i) the decrease of the peak area of *O*-benzylhydroxylamine corresponded to the amount of aldehyde engaged in the reaction, and (ii) the method was independent of the aldehyde. Different amounts of aldehyde **4** or **5** (C_{ald}) were reacted with **1** at 1.5 mM (C_0) and the reaction mixtures were analysed by RP-HPLC. In Figure 5, the peak area of *O*-benzylhydroxylamine **1** was plotted as a function of $C_0 - C_{ald}$, i.e. the expected *O*-benzylhydroxylamine concentration in the reaction mixture assuming the total conversion of the aldehydes **4** or **5** into their corresponding *O*-benzyloximes. For comparison, the peak area of known concentrations of **1** were also included in Figure 5. The relationship between peak area and $C_0 - C_{ald}$ or the known concentration of **1** was linear and the regression lines were not significantly different ($p < 0.05$), demonstrating that the quantity of **1** consumed in the reaction amounts to the quantity of aldehyde in the sample. The regression line obtained using glyoxylic acid **4**, which requires 21 experiments and RP-HPLC analyses, can be used for the determination of glyoxylyl-peptide concentration. The standard line is valid whatever the structure of the glyoxylyl-peptide. The accuracy (proximity to the true value), the precision (random error) and reproducibility (between-run precision) at 0.75 mM were 0.7, 1.3 and 2.2% respectively, highlighting the usefulness of the method [21]. One experiment requires about 40 nmoles of aldehyde only.

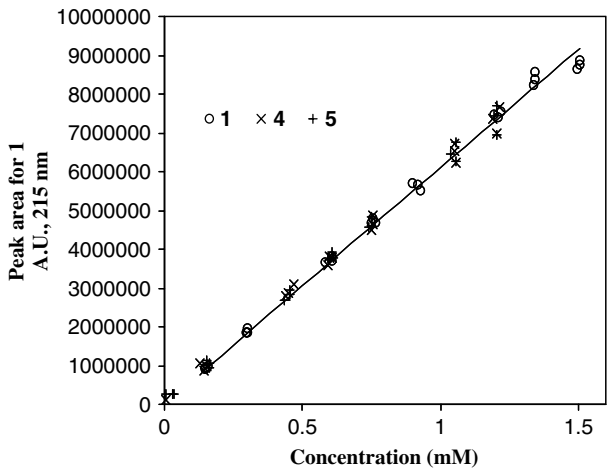

Figure 5 *O*-Benzylhydroxylamine **1** ($C_0 = 1.5$ mM) was reacted with different amounts of aldehyde **4** (×) or **5** (+) (concentration C_{ald}). The reaction mixture was analysed by RP-HPLC and the peak area of **1** was plotted as a function of $C_0 - C_{ald}$. The data obtained for known concentrations of **1** (○) and the corresponding regression line were also presented for comparison. The regression lines for **4** and **5** were omitted for clarity. Three independent experiments were performed for each concentration.

The data in Figure 4 can be presented differently by plotting $(1 - A/A_0)C_0$ as a function of the known aldehyde concentration (Figure 6). The relationship between $(1 - A/A_0) \times C_0$ and aldehyde concentration is very close to the bisecting line $y = x$. Now, only two HPLC analyses are necessary to determine aldehyde concentration and the accuracy of the method is of course lower than when using the standard line described above, especially for concentrations below 0.5 mM. In this case, the quantity of aldehyde used in a second analysis can be adjusted so as to be above 0.5 mM.

Application to Peptide 2b

Peptide **2b** (Figure 2) was synthesised by treating the corresponding seryl precursor with sodium periodate [8]. A solution of peptide **2b** was analysed as described above. $(1 - A/A_0)C_0$ was equal to 0.58 mM, which is close to the peptide concentration determined by quantitative amino acid analysis after acid hydrolysis (0.56 mM).

CONCLUSION

We have described a simple method that allows the determination of glyoxylyl-peptide concentration. The method, based on oxime formation with *O*-benzylhydroxylamine and RP-HPLC analysis, was validated using 4-hydroxybenzaldehyde or glyoxylic acid. The analysis of n compounds requires $n + 1$ RP-HPLC analyses and about 40 nmoles of each aldehyde compound. The duration of one HPLC run is only a few minutes due to the low retention time of *O*-benzylhydroxylamine.

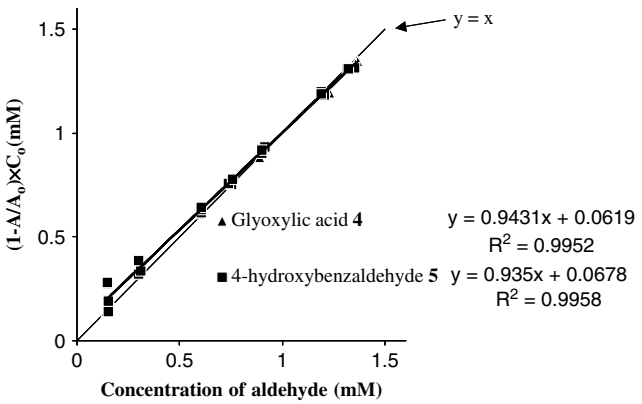

Figure 6 $(1 - A/A_0) \times C_0$ as a function of glyoxylic acid **4** (triangles) or 4-hydroxybenzaldehyde **5** (squares) concentration. A is the area of *O*-benzylhydroxylamine **1** after reaction with aldehyde **4** or **5**, A_0 is the area of **1** at $C_0 = 1.5$ mM. The regression lines are indicated in the figure. The bisecting line is included for comparison.

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