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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 127–136
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

LC-UV/MS characterization and DOE optimization of the iodinated peptide obestatin

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Received 5 July 2007; accepted 4 October 2007
Available online 13 October 2007

Abstract

The aim of this study was to investigate the direct iodination of the recently discovered peptide obestatin by LC-UV/ESI ion trap MS analysis. The influence of selected reaction parameters on obestatin iodination by chloramine-T, Iodo-Gen® and lactoperoxidase was investigated by experimental design. Different responses, *i.e.* species percentage and yield, peptide recovery and iodination yield were evaluated. Mono-up till tetra-iodinated species are possible depending on the reaction conditions with electrophilic substitutions occurring at Tyr^{16} and His^{19} as confirmed by LC/MS/MS. The two possible mono-iodinated obestatin isomers, *i.e.* [I_1-Tyr^{16}]-obestatin and [I_1-His^{19}]-obestatin, could be chromatographically separated. Several significant main and quadratic effects, and interaction of factors were observed from which optimum conditions for a specific response could be derived. The highest impact on the response surface diagrams was overall attributed to the amount of iodide added. Synthesis methods were compared relative to the different response factors: lactoperoxidase was found to be the overall most robust iodination technique, and also gave the highest mono-iodinated species yield. The applicability of our research was demonstrated by non-carrier-added ^{125}I-radioiodination. To our knowledge, this is the first time an LC separation of mono-iodinated peptide isomers has been reported.

Keywords: Peptide iodination; Obestatin; Chloramine-T; Iodo-Gen®; Lactoperoxidase; Design of experiments (DOE); ESI-ion trap MS/MS

1. Introduction

Obestatin is a very recently discovered functional peptide that was recognized as an appetite suppressing hormone by Zhang et al. [1]. It is encoded by the same gene as ghrelin, *i.e.* an appetite stimulating peptide hormone that was discovered in 1999 by Kojima et al. [2]. Although the possible receptors of both hormones are being investigated (GPR39 and G-protein coupled membrane receptor, resp.), the mechanism of action of these bioactive peptides still remains unclear [3–5]. Because the original findings by Zhang on obestatin binding and activation of GPR39 receptors *in-vitro* cannot be reproduced, it was hypothesized that obestatin may lose bioactivity after iodination as up to four iodine molecules can be incorporated into the obestatin peptide [5,6].

The continually rising prevalence of overweight and obesity in the United States and Europe (especially in children and adolescents) [7,8] makes obesity a leading public health problem. Hence, obestatin currently receives much attention in view of the development of a novel class of drugs for the treatment of eating disorders [9].

Radioiodination (*i.e.* incorporation of radioactive iodine such as ^{123}I, ^{125}I or ^{131}I into a molecule) is a technique commonly

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doi:10.1016/j.jpba.2007.10.005

used on peptides for *in-vitro* radioligand investigations as well as for medical imaging and therapy. Several direct and indirect iodination procedures currently exist. The following direct labelling techniques are considered to be the most widely used: chloramine-T (CAT) [10], Iodo-Gen® (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) [11] and lactoperoxidase [12]. These procedures result in radioiodine substituted tyrosine and histidine amino acid residues depending on the reaction parameters. Mono- and higher iodinated peptides can be formed which may exhibit different biological activities and/or pharmacokinetic properties [13,14]. Differences are also observed between these iodinated peptides and the non-iodinated native peptide (NIP). While this is expected to be of a lesser concern for proteins, where iodinations minimally change the structure of the protein, this is not so for peptides due to their smaller size, and hence the greater influence of the iodination on the properties of the peptide.

Until now, no multiple-response optimizations using experimental designs on peptide iodinations have been described, nor an analytical characterization of the iodinated obestatin species obtained and used in radioligand studies despite the pharmacological research importance. To our best knowledge, chromatographic separation of the mono-iodinated compounds derived from a single peptide containing both tyrosine and histidine amino acid residues has not been published yet. For the current study, response surface designs were applied on mouse obestatin (FNAPFDVGIKLSGAQYQQHGRAL-NH_2) using three direct iodination techniques. This peptide consists of 23 amino acid residues and contains two aromatic amino acid residues in its sequence which are available for direct iodination: one tyrosyl and one histidyl group. The effects of different reaction parameters, such as pH, reaction time, amount of iodide and chloramine-T/iodide ratio were evaluated using Draper-Lin (chloramine-T) and Box-Behnken design (Iodo-Gen® and lactoperoxidase). The effect on the amounts of mono-, di-, tri- and tetra-iodinated obestatin species formed (percentage MIP, DIP, 3IP, and 4IP, resp.), peptide recovery and overall iodination yield were determined by HPLC-UV and LC-UV/MS. HPLC-PDA/Fluorescence and LC-UV/MS^2 were used for the analytical characterisation.

2. Materials and methods

2.1. Materials

Mouse obestatin was obtained from California Peptide Research (Napa, CA, USA). Iodo-Gen® precoated tubes (50 μg/tube) were purchased from Pierce (Rockford, IL, USA). Lactoperoxidase from bovine milk was purchased from Sigma (St. Louis, CA, USA). HPLC gradient grade acetonitrile was purchased from Rathburn (Walkerburn, UK). LC-MS grade formic acid was purchased from Fluka (Buchs, Switzerland). $Na^{125}I$ in dilute NaOH solution (3700 MBq/mL) was purchased from MDS Nordion (Fleurus, Belgium). Water was purified in the laboratory by distillation of demineralised water. All other reagents were purchased from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Peptide lyophilisation

Prior to iodination, obestatin was dissolved in aqueous trifluoroacetic acid (0.1% w/v) at about 1 mg/mL, dispensed into microtubes, and lyophilized using a Lyovac GT4 pre-cooled shelve freeze-dryer (Leybold, Cologne, Germany).

2.2.2. Obestatin iodination using chloramine-T

The method used is based on the procedure described by Hunter and Greenwood, 1962 [10].

To a solution containing 10 nmol of mouse obestatin in 60 μL of phosphate buffer (pH ranging from 6.9 to 8.5: as prescribed, 200 mM), 10 μL of chloramine-T solution (2.9–105.0 nmol: as prescribed), and 10 μL of sodium iodide solution (2.5–36.5 nmol: as prescribed) were subsequently added. The mixture was mixed, and allowed to react at room temperature for the prescribed time ranging from 20 to 60 s. The reaction was stopped by adding 10 μL of sodium metabisulphite solution (with concentration matched to that of chloramine-T).

2.2.3. Obestatin iodination using Iodo-Gen®

The procedure was described by Salacinski et al., 1981 [11].

Ten microlitres of sodium iodide solution (2.5–40 nmol: as prescribed) was transferred into a Iodo-Gen® coated tube containing 10 nmol of mouse obestatin in 100 μL of phosphate buffer (pH ranging from 7.0 to 8.5: as prescribed, 130 mM). After the prescribed time ranging from 5 to 15 min at room temperature, the reaction was stopped by removing the solution from the tube.

2.2.4. Obestatin iodination using lactoperoxidase

The applied method is based on the procedure described by Marchalonis, 1969 [12].

To a solution containing 10 nmol of mouse obestatin in 100 μL of phosphate buffer (pH ranging from 7.0 to 8.5: as prescribed, 130 mM), 10 μL of sodium iodide solution (2.5–40 nmol: as prescribed), 10 μL of lactoperoxidase solution (0.067 mg/mL) and 10 μL of hydrogen peroxide (0.005% w/v) were subsequently added. After the prescribed time ranging from 2 to 10 min at room temperature, a second amount of hydrogen peroxide (10 μL, 0.005% w/v) was added, and again allowed to react at room temperature until 10 μL of sodium metabisulphite solution (1.2 mg/mL) was added to stop the reaction at the prescribed time.

2.2.5. Obestatin ^{125}I radiolabelling

Twenty nanomoles of mouse obestatin in 160 μL of phosphate buffer (pH 7.4, 130 mM) was mixed with 40 μL of $Na^{125}I$ solution (148 MBq) and transferred into a Iodo-Gen® coated tube. After 10 min at room temperature, the reaction was stopped by removing the solution from the tube.

2.2.6. Liquid chromatography

The LC-UV/MS apparatus consisted of a Spectra System SN4000 interface, a Spectra System SCM1000 degasser, a

Table 1
Chosen levels of the experimental factors

Variable	Code	Low Level	Centerpoint	High Level
Draper-Lin cube-star design for Chloramine-T iodination technique				
pH	A	7.30	7.70	8.10
Reaction time [s]	B	30	40	50
Amount of NaI [nmol/10 nmol of obestatin]	C	11.00	19.5	28.00
CAT/NaI ratio [nmol of CAT/10 nmol of NaI]	D	13.50	25.5	37.50
Box-Behnken design for Iodo-Gen® iodination technique				
pH	A	7.00	7.75	8.50
Reaction time [min]	B	5	10	15
Amount of NaI [nmol/10 nmol of obestatin]	C	2.50	21.25	40.00
Box-Behnken design for lactoperoxidase iodination technique				
pH	A	7.00	7.75	8.50
Reaction time [min]	B	2	6	10
Amount of NaI [nmol/10 nmol of obestatin]	C	2.50	21.25	40.00

Spectra System P1000XR pump, a Spectra System AS3000 autosampler and a Finnigan LCQ Classic ion trap mass spectrometer in positive ion mode (all Thermo, San José, CA, USA) equipped with a SPD-10A UV–vis detector (Shimadzu, Kyoto, Japan) and Xcalibur 1.2 software (Thermo, San José, CA, USA) for data acquisition. ESI was conducted using a needle voltage of 4.5 kV. Nitrogen was used as the sheath and auxiliary gas with the heated capillary set at 270 °C. CID experiments employed helium with a collision energy at 22.5%.

The HPLC-PDA/FL apparatus consisted of a Waters Alliance 2695 separations module, a Waters 2996 photodiode array detector and a Waters 2475 multi-wavelength fluorescence detector with Empower 2 software for data acquisition (all Waters, Milford, MA, USA).

The radio-HPLC apparatus consisted of a LaChrom Elite L-2130 pump with degasser, a LaChrom Elite L-2300 column oven, a LaChrom Elite L-2400 UV detector set at 215 nm (all Hitachi, Tokyo, Japan), a Rheodyne 7725i manual injector with 100 μL sample loop (Rheodyne, Rohnert Park, CA, USA) and a Berthold LB500 HERM radioactivity detector (Berthold Technologies, Bad Wildbad, Germany) equipped with EZChrom Elite version 3.1.7 software for data acquisition (Scientific Software, Pleasanton, CA, USA).

LC separations for the determination of the NIP, MIP, DIP, 3IP and 4IP species were performed using a Vydac P&P C_{18} (250 mm × 4.6 mm i.d., 300 Å, 5 μm particle size) column (Grace Vydac, Hesperia, CA, USA) in an oven set at 30 °C, with a mobile phase consisting of A) 0.1% w/v formic acid in water, and B) 0.1% w/v formic acid in acetonitrile. A linear gradient was employed as follows: 0 min, 82% A; and 60 min, 68% A. The flow rate was set at 1.0 mL/min.

LC separations for the determination of His-MIP and Tyr-MIP were performed using a Vydac Diphenyl (250 mm × 4.6 mm i.d., 300 Å, 5 μm particle size) column (Grace Vydac, Hesperia, CA, USA) in an oven set at 30 °C, with a mobile phase consisting of A) 0.1% w/v formic acid in water, and B) 0.1% w/v formic acid in acetonitrile. The mono-iodinated obestatin compounds were eluted using an isocratic run with 80% A for 60 min at 1.0 mL/min.

2.2.7. Experimental design

For the chloramine-T experiments, a randomized Draper-Lin cube-star design [15] was used to investigate the effects of the following reaction parameters on the iodination of 10 nmol of obestatin: pH, reaction time, amount of iodide, and CAT/iodide ratio. The effects of these four factors were investigated in 20 runs, including four centerpoints (see Table 1).

For Iodo-Gen® and lactoperoxidase experiments, a randomized Box-Behnken design [16] with 15 runs was performed to investigate the effects of the following three reaction parameters: pH, reaction time and amount of iodide. The parameters were investigated on three levels (see Table 1).

StatGraphics Plus software version 5.1 (StatPoint, Herndon, VA, USA) was applied for generating the experimental design as well as analysis thereof. The regression coefficients were calculated based on the quadratic polynomial Eq. (1) for the experimental data:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_i \sum_j \beta_{ij} x_i x_j + \sum_i \beta_{ii} x_i^2 \quad (1)$$

where Y is the predicted response associated with each factor level combination; β_0 is the constant term; β_i, β_{ij}, β_{ii} are the regression coefficients; and x_i, x_j are the independent variables or reaction parameters. For each iodination technique, statistically non-significant [$P = 0.10$] effects and interactions were deleted from the models, and the final reduced models recalculated with significant coefficients only.

2.2.8. Response factors

Mono-iodinated obestatin (MIP) yield was expressed as the percentage of nmole MIP formed with reference to the amount of limiting reactant in nmol, *i.e.* obestatin at 10 nmol, except for those cases where the amount of sodium iodide or chloramine-T is lower.

Percentage of mono-, di-, tri- and tetra-iodinated obestatin formed (MIP%, DIP%, 3IP% and 4IP%, respectively) was calculated as the percentage of nmole species formed with reference to 10 nmol of native obestatin (non-iodinated peptide or NIP).

Peptide recovery was calculated as the sum of NIP%, MIP%, DIP%, 3IP% and 4IP%.

Table 2
Iodinated obestatin species formed for chloramine-T technique

Draper-Lin cube-star design									
Run	Factor A	Factor B	Factor C	Factor D	NIP (nmol)	MIP (nmol)	DIP (nmol)	3IP (nmol)	4IP (nmol)
1	7.30	50	11.00	37.5	1.505	2.709 [98.6]	4.274	Not detected	Not detected
2	7.30	30	28.00	13.5	Not detected	Not detected	8.306	2.998	0.476
3	8.10	30	28.00	37.5	Not detected	Not detected	4.153	3.521	1.618
4	7.30	50	28.00	37.5	Not detected	Not detected	2.769	2.712	Not detected
5	7.30	30	11.00	13.5	2.167	3.912 [97.9]	3.130	Not detected	Not detected
6	7.70	40	19.50	25.5	Not detected	Not detected	1.866	Not detected	Not detected
7	8.10	50	11.00	13.5	4.875	4.274 [97.9]	0.602	Not detected	Not detected
8	8.10	30	11.00	37.5	0.963	1.445 [98.2]	1.926	Not detected	Not detected
9	8.10	50	28.00	13.5	Not detected	Not detected	1.324	Not detected	Not detected
10	7.70	40	19.50	25.5	Not detected	Not detected	7.223	1.618	0.381
11	7.70	40	2.50	25.5	9.931	0.482 [100]	Not detected	Not detected	Not detected
12	6.90	40	19.50	25.5	Not detected	Not detected	8.246	0.761	Not detected
13	7.70	40	19.50	49.5	Not detected	Not detected	6.982	Not detected	Not detected
14	7.70	40	19.50	25.5	Not detected	Not detected	9.029	0.523	Not detected
15	8.50	40	19.50	25.5	0.542	4.093 [97.2]	3.672	Not detected	Not detected
16	7.70	40	19.50	25.5	Not detected	Not detected	7.584	0.809	Not detected
17	7.70	20	19.50	25.5	Not detected	0.421 [95.9]	1.926	Not detected	Not detected
18	7.70	40	36.50	25.5	Not detected	Not detected	Not detected	4.425	2.665
19	7.70	40	19.50	1.5	7.704	2.287 [94.9]	Not detected	Not detected	Not detected
20	7.70	60	19.50	25.5	Not detected	Not detected	0.421	Not detected	Not detected

For MIP, the percentage Tyr-MIP is given between square brackets.

The iodination yield was calculated as: [MIP% + 2 × DIP% + 3 × 3IP% + 4 × 4IP%] × amount of peptide (*i.e.* 10 nmol)/[amount of limiting reagent in nmol]. The limiting reagent can be either sodium iodide, chloramine-T or hydrogen peroxide.

3. Results

For each experiment, the nmole amounts of non-, mono-, di-, tri-, and tetra-iodinated obestatin recovered (NIP, MIP, DIP, 3IP and 4IP, respectively) were determined using relative response factors (RRF) for 3IP and 4IP (both taken equal to 1.265). The RRF values were calculated based on selected chromatograms from initial screening tests showing a high total peptide peak area (which were considered to correspond with 100% peptide recovery). The percentage Tyr-MIP was calculated by normalization with reference to the His-MIP and Tyr-MIP peak areas. The results for each run were tabulated in Tables 2–4 for chloramine-T, Iodo-Gen®, and lactoperoxidase, respectively.

3.1. Separation of [I_1-Tyr^{16}]-obestatin (Tyr-MIP) and [I_1-His^{19}]-obestatin (His-MIP)

Attempts to separate the two MIP-obestatin positional isomers [I_1-Tyr^{16}]-obestatin and [I_1-His^{19}]-obestatin on a Vydac C_{18} HPLC column were not completely satisfying, with only low separation factor α ($=k_2/k_1$) in isocratic mode. However, using a Vydac Diphenyl HPLC column in isocratic mode with 20% v/v of acetonitrile in the mobile phase, His-MIP and Tyr-MIP could be sufficiently separated with a separation factor α of 1.2 (Fig. 1). The His-MIP peak demonstrates similar UV and fluorescence spectra (*i.e.* absorption maximum at 275 nm; fluorescence for $\lambda_{Ex} = 230$ nm and $\lambda_{Em} = 300$ nm) when compared to the native obestatin, whereas the Tyr-MIP peak shows different spectral properties (*i.e.* absorption maximum at 284 nm [6]; no fluorescence). The identity of both compounds was confirmed by tandem mass spectrometry. Analysis of the samples for all three iodination techniques revealed that consistently only a small fraction of the mono-iodinated obestatin formed was present as [I_1-His^{19}]-obestatin (*i.e.* less than 10%).

3.2. LC/MS/MS analysis of obestatin and iodinated species

The presence of obestatin and its iodinated species in the reaction mixtures was confirmed by LC/ESI/MS analysis based on molecular mass.

LC/MS/MS analysis of mono-iodinated obestatin was consistent with iodine incorporation at Tyr^{16} as predominant site of labelling. The CID spectrum of this MIP species (Fig. 2) resulted in the generation of iodination-site-specific product ions at *m/z* 549.2 (y_8), 552.3 (y_5), 680.4 (y_6), 773.4 (b_{15}), 808.5 (y_7), 917.9 (b_{16}), 981.9 (b_{17}), 1045.9 (b_{18}), 1097.4 (y_8), and 1834.8 (b_{16}).

Using collision-induced dissociation (CID) analysis on di-, tri- and tetra-iodinated obestatin, Tyr^{16} and His^{19} were identified as sites of iodination by all three direct labelling techniques.

3.3. Iodination models

For all three iodination techniques, polynomial equations for the reduced models were generated for the seven different responses. The goodness-of-fit (R-squared) for these models and the statistically significant terms obtained are overviewed in Table 5.

No models were derived separately for [I_1-Tyr^{16}]-obestatin and [I_1-His^{19}]-obestatin as their relative formation was found to be not significantly dependent upon the reaction parameters, and hence, overall described by MIP% and MIP yield.

Table 3
Iodinated obestatin species formed for Iodo-Gen® technique

Box-Behnken design								
Run	Factor A	Factor B	Factor C	NIP (nmol)	MIP (nmol)	DIP (nmol)	3IP (nmol)	4IP (nmol)
1	7.75	10.00	21.25	4.959	2.040 [96.4]	2.468	0.656	0.598
2	8.50	5.00	21.25	3.843	3.102 [96.7]	3.043	0.517	0.577
3	7.75	5.00	2.50	7.171	1.746 [100]	0.518	0.233	0.031
4	7.00	15.00	21.25	1.974	1.744 [100]	4.339	0.809	1.915
5	7.75	10.00	21.25	4.599	2.026 [100]	2.590	0.645	0.696
6	8.50	10.00	40.00	2.771	2.440 [97.8]	2.781	1.159	2.127
7	8.50	10.00	2.50	6.349	1.534 [97.9]	0.412	0.247	Not detected
8	7.75	10.00	21.25	4.661	1.943 [98.9]	2.845	0.795	0.907
9	7.00	10.00	2.50	7.189	1.487 [99.4]	0.569	0.121	0.040
10	7.75	15.00	2.50	7.856	1.463 [100]	0.510	0.135	Not detected
11	7.00	10.00	40.00	0.349	0.639 [100]	2.694	1.116	5.272
12	7.00	5.00	21.25	3.573	2.312 [100]	4.061	0.989	1.001
13	7.75	5.00	40.00	4.584	2.397 [95.3]	2.483	0.991	1.058
14	7.75	15.00	40.00	4.552	1.577 [94.0]	1.948	1.018	2.062
15	8.50	15.00	21.25	3.119	2.359 [95.6]	3.789	1.017	1.283

For MIP, the percentage Tyr-MIP is given between square brackets.

Table 4
Iodinated obestatin species formed for lactoperoxidase technique

Box-Behnken design								
Run	Factor A	Factor B	Factor C	NIP (nmol)	MIP (nmol)	DIP (nmol)	3IP (nmol)	4IP (nmol)
1	7.75	6.00	21.25	2.059	3.807 [96.6]	3.917	Not detected	Not detected
2	8.50	2.00	21.25	5.266	2.513 [100]	0.702	Not detected	Not detected
3	7.75	2.00	2.50	7.414	1.346 [100]	0.088	Not detected	Not detected
4	7.00	10.00	21.25	Not detected	1.430 [96.4]	8.353	0.561	Not detected
5	7.75	6.00	21.25	1.067	3.145 [97.6]	5.217	Not detected	Not detected
6	8.50	6.00	40.00	5.267	2.782 [96.0]	0.770	Not detected	Not detected
7	8.50	6.00	2.50	8.095	0.670 [100]	Not detected	Not detected	Not detected
8	7.75	6.00	21.25	0.947	3.741 [96.6]	5.396	Not detected	Not detected
9	7.00	6.00	2.50	7.816	2.590 [100]	Not detected	Not detected	Not detected
10	7.75	10.00	2.50	6.669	1.561 [100]	Not detected	Not detected	Not detected
11	7.00	6.00	40.00	Not detected	1.256 [97.2]	9.160	1.412	Not detected
12	7.00	2.00	21.25	Not detected	1.229 [97.7]	9.080	0.617	Not detected
13	7.75	2.00	40.00	0.948	3.581 [96.5]	5.388	0.374	Not detected
14	7.75	10.00	40.00	1.050	3.436 [95.9]	5.451	0.376	Not detected
15	8.50	10.00	21.25	2.345	4.388 [96.2]	1.670	Not detected	Not detected

For MIP, the percentage Tyr-MIP is given between square brackets.

Table 5
ANOVA results overview for reduced models

Response factors	Iodination technique					
	Chloramine-T		Iodo-Gen®		Lactoperoxidase	
	R^2 (%)	Significant effects and interactions ($P \leq 0.100$)	R^2 (%)	Significant effects and interactions ($P \leq 0.100$)	R^2 (%)	Significant effects and interactions ($P \leq 0.100$)
MIP yield	91.9	D^-, DD^+, C^-, AB^-, A^+, AA^+, BD^+	99.8	C^-, CC^+, A^+, B^-, AC^+, BB^+	75.7	C^-, AC^+, CC^+
MIP%	73.5	C^-, A^+, AA^+, BD^+	96.4	A^+, CC^-, AC^+, B^-, BB^+	70.4	C^+, AA^-, AC^+
DIP%	74.5	CC^-, BB^-, D^+	96.3	CC^-, C^+, AA^+, BB^+	95.8	A^-, C^+, CC^-, AC^-
3IP%	83.6	C^+, CC^+	96.4	C^+, AB^+, CC^-	96.5	A^-, C^+, AC^-, AA^+, CC^+
4IP%	81.1	C^+, CC^+	87.4	C^+, AC^-, A^-, AA^+	–	–
Peptide recovery	70.4	BC^-, BB^-	87.2	AC^+, CC^-, AA^-, BB^+	89.8	A^-, C^+
Iodination yield	58.0	BB^-	92.1	C^-, CC^+, AA^+	98.4	A^-, C^-, AB^+, CC^-

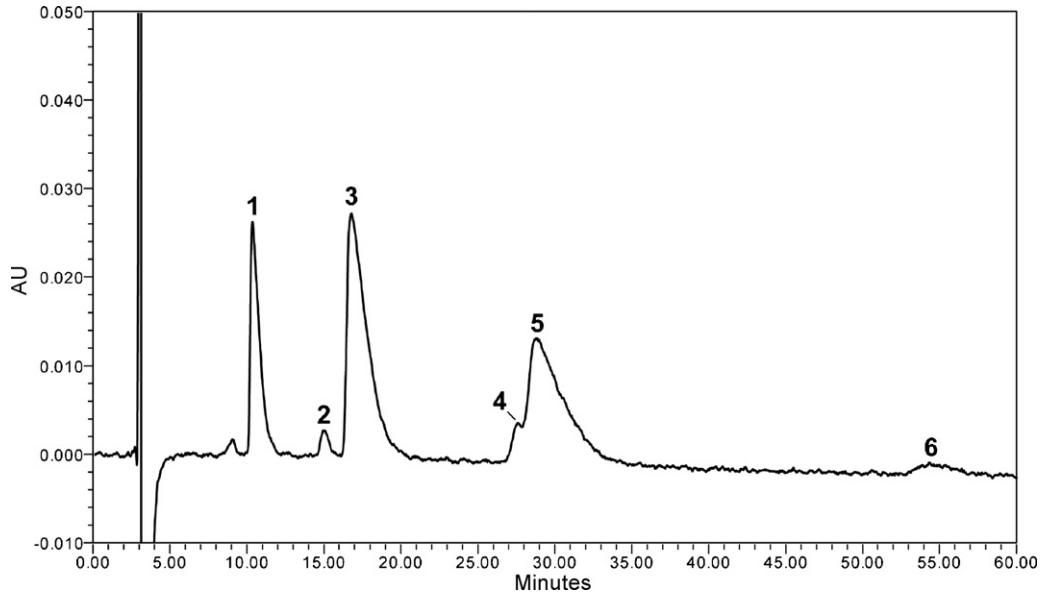

Fig. 1. Typical chromatogram (UV @ 215 nm) obtained on iodinated obestatin using a Vydac Diphenyl 300 Å 5 μm (250 × 4.6 mm i.d.) column with chromatographic conditions as described in Section 2.2. Peaks at 10.4, 15.0, 16.8, 27.6, 28.8 and 55.0 min correspond to (1) NIP, (2) His-MIP, (3) Tyr-MIP, (4) probably DIP with mono- and/or di-iodinated histidyl residue, (5) di-Tyr-DIP and (6) 3IP, respectively.

For the chloramine-T technique, the B, AC, AD and CD terms were found statistically not significant, and hence excluded from the models used for the calculation of the optimal factor values. The iodination yields obtained range from 4% to 107% (*i.e.* run 20 and 10, respectively). The peptide recoveries found lie within 4% and 118% (*i.e.* run 20 and 2, respectively).

For the Iodo-Gen® technique, the BC interaction term was not significant, and thus excluded from the models. The iodination yields obtained range from 36.4% to 144.1% (*i.e.* run 13 and 3, respectively). The peptide recoveries found lie within 48.0% and 109.4% (*i.e.* run 11 and 12, respectively).

For the lactoperoxidase technique, the non-significant B, BB and BC terms were excluded from the models. The iodination yields obtained range from 15% to 104% (*i.e.* run 6 and 9, respectively). The peptide recoveries found lie within 82% and 118% (*i.e.* run 10 and 11, respectively).

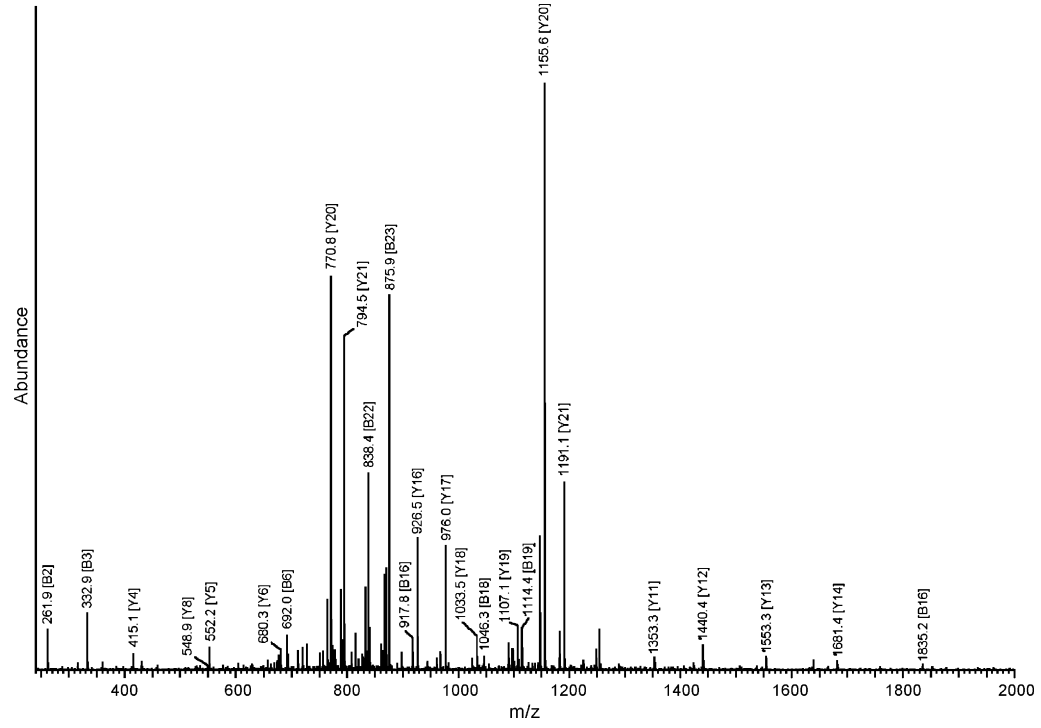

Fig. 2. LC/MS/MS analysis of [I_1-Tyr^{16}]-obestatin prepared using the chloramine-T technique. CID was conducted on $[M+3H]^{3+}$ at *m/z* 882.

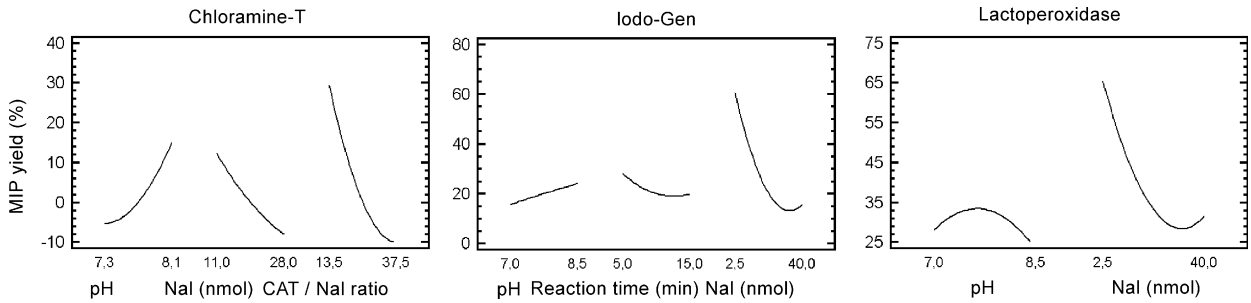

Fig. 3. Main effects plot showing the effect of reaction conditions on the formation of mono-iodinated obestatin.

3.3.1. Formation of mono-iodinated obestatin

Main effect plots for the MIP yield were generated for all three iodination techniques as shown in Fig. 3. The MIP yield models were applied to non-carrier-added ^{123}I or ^{125}I radioiodination, and optimum reaction conditions were calculated for an extrapolated 0.25 nmol iodide amount (which is equivalent to 18.5 MBq of ^{125}I). Under these conditions, for the chloramine-T technique, optimum MIP yield was obtained for a reaction time of 21 s at pH 8.5, with CAT/iodide ratio as low as possible. For the Iodo-Gen® technique, optimum MIP yield was obtained at pH 8.5 and a reaction time of 5 min. For the lactoperoxidase technique, optimum MIP yield was obtained at pH 7.0.

Response surface plots for MIP% were generated for all three iodination techniques as shown in Fig. 4. In the experimental range of reaction variables, the highest MIP% values were observed for chloramine-T and lactoperoxidase iodination techniques: up to 43% and 44%, respectively (while maximum 31% for Iodo-Gen®). For chloramine-T, optimum MIP% was determined for 1 molar equivalent of sodium iodide at pH 8.5, and a reaction time of 20 s with 1 molar equivalent of CAT. For the lactoperoxidase technique, optimum MIP% was obtained for 3.8 molar equivalents of sodium iodide, and pH 8.4. For the Iodo-Gen® technique, optimum MIP% was obtained at pH 8.5 for a reaction time of 5 min.

3.3.2. Formation of di-iodinated obestatin

For all three techniques, a strong negative second order effect of sodium iodide amount was found (*i.e.* clear maximum DIP% for intermediate level of sodium iodide). Optimum, significant reaction conditions were again calculated from the data for each iodination technique. For chloramine-T, optimum DIP% was determined for 2.5 molar equivalents of sodium iodide, a reaction time of 23 s, with 3.8 molar equivalents of CAT relative to the amount of sodium iodide. For the Iodo-Gen® technique, optimum DIP% was obtained for 2.6 molar equivalents of sodium iodide. For the lactoperoxidase technique, optimum DIP% was obtained for 3.9 molar equivalents of sodium iodide, and pH 7.0. Highest experimental DIP% values were observed for chloramine-T and lactoperoxidase iodination techniques.

3.3.3. Formation of tri-iodinated obestatin

For chloramine-T and Iodo-Gen® technique, 3IP% optima were determined for 3.7 and 3.9 molar equivalents of sodium iodide, respectively. Other effects of the reaction variables were found to be statistically insignificant. For the lactoperoxidase technique, optimum 3IP% was obtained for 4.0 molar equivalents of sodium iodide, and pH 7.0. Again, reaction time was found to be statistically insignificant.

3.3.4. Formation of tetra-iodinated obestatin

As tetra-iodinated obestatin could not be detected in the lactoperoxidase runs using UV at 215 nm, no model was deducted. Nevertheless, very small amounts were found using mass spectrometry. These findings are expected as only 3 molar equivalents of hydrogen peroxide (with reference to obestatin) were used for each reaction.

For chloramine-T, optimum 4IP% was determined for 4.1 molar equivalents of sodium iodide. For the Iodo-Gen® technique, optimum 4IP% was obtained with 4.0 molar equivalents of sodium iodide at pH 7.0.

3.3.5. Peptide recovery

For the lactoperoxidase technique, a statistically significant strong negative first order effect of pH was observed ($P = 0.0001$). For Iodo-Gen®, a statistically significant very strong negative second order effect of sodium iodide amount was observed ($P = 0.0198$), indicating an optimum.

For chloramine-T, statistically significant strong negative second order effect of reaction time and a very strong negative interaction between reaction time and sodium iodide amount were observed ($P = 0.0328$ and 0.0327, respectively). Therefore,

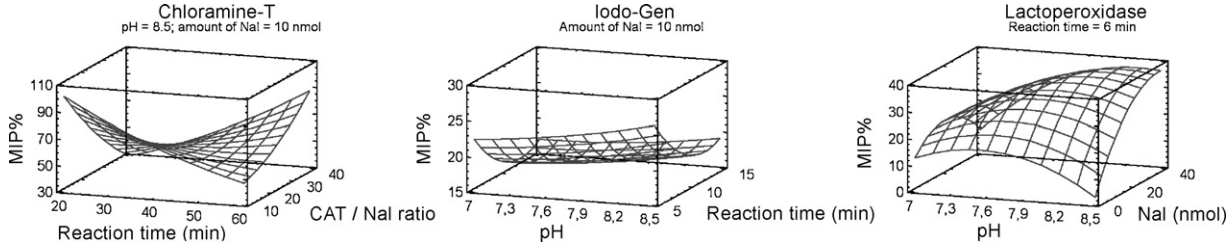

Fig. 4. Response surface plot (3D) showing the effect of reaction conditions on the percentage of mono-iodinated obestatin with reference to the native peptide.

when high NaI amounts are used, the peptide recovery decreases in function of reaction time. For low NaI amounts, the peptide recovery improves as the reaction time increases.

3.3.6. Iodination yield

For chloramine-T, a negative second order effect of reaction time was observed ($P = 0.0573$). For Iodo-Gen®, a negative first order and positive second order effect of sodium iodide were observed ($P = 0.0003$ and 0.0393, respectively). Using the lactoperoxidase technique, several statistically significant effects and interactions were observed which could not be entirely explained by the close relationship between iodination yield and peptide recovery, *e.g.* negative first order effect of sodium iodide ($P = 0.0033$).

3.4. Radio-HPLC analysis of obestatin labelled with ^{125}I using Iodo-Gen®

Not only for carrier-added iodination, but also for non-carrier-added labelling, the models and analytics can be used. Evidence of the formation of non-negligible amounts of di-, tri- and tetra-iodinated obestatin by ^{125}I non-carrier-added direct labelling is given in Fig. 5, showing a radio-HPLC chromatogram obtained on obestatin iodinated at pH 7.4 using 18.5 MBq of $Na^{125}I$ and Iodo-Gen® as oxidizing agent. The applied conditions were derived from our model, predicting a maximum MIP yield. Experimentally, a MIP yield of 80% was obtained.

4. Discussion

4.1. LC/MS/MS analysis of obestatin and iodinated species

LC/MS allowed unambiguously the characterization of the iodination-degree of the eluting iodinated peaks, which were clearly separated on the gradient-based system: *m/z* values of 839.9, 881.6, 924.1, 965.7, and 1007.4, corresponding to NIP, MIP, DIP, 3IP and 4IP, respectively ($z = 3$), could be clearly attributed to the different peaks.

The possibility of iodinating tyrosyl and histidyl amino acid residues was already demonstrated for several peptides and iodination techniques [17,18]. Although, obestatin could be labelled at both Tyr^{16} and His^{19} using all iodination techniques tested, a clear preference towards Tyr^{16} was observed under all our experimental conditions. In the tandem mass spectra, the mass-to-charge ratios of the fragment ions corresponding to b_{16}, b_{17}, y_6 and y_5 (amino acid residues 1–16, 1–17, 18–23 and 19–23, respectively) are considered to be highly useful for identifying the iodinated amino acids.

4.2. Synthesis evaluation

Once the peak assignments were performed, quantification of the cold carrier-added synthesis products was done using UV detection. From the assay results, an empirical mathematical model was calculated. Overall, the models obtained for Iodo-Gen® gave the highest number of significant effects and interactions (*e.g.* MIP percentage: $n = 5$). This means that the outcome for this technique is affected by small variations in operational method parameters. In addition, our results clearly demonstrate that a reaction time of 5 min or less is sufficient.

The models obtained for MIP yield using chloramine-T gave the highest number of significant effects and interactions ($n = 7$). Hence, for optimum MIP yield, all reaction variables should be carefully chosen and controlled within a rather narrow operational domain.

On average, the models obtained for the lactoperoxidase technique gave the lowest number of significant effect and interactions on the formation of mono-iodinated obestatin (both MIP% and MIP yield: $n = 3$). Hence, this method is considered to be more robust, although the pH has a significant influence on the outcome.

The mono-iodinated peptide is the most important species for radioligand and ADME studies as its molecular structure is the closest to the native obestatin. One of the most important parameters on the formation of MIP is attributed to the amount of

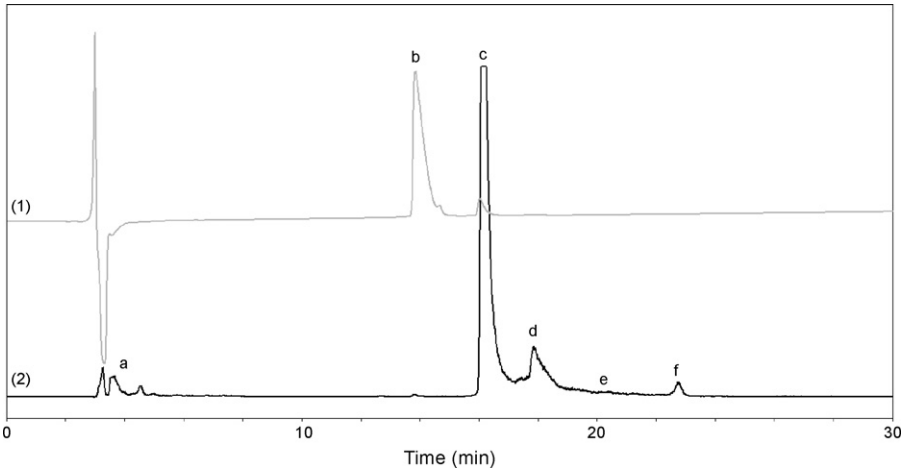

Fig. 5. Radio-HPLC chromatogram obtained on non-carrier-added ^{125}I obestatin prepared by the Iodo-Gen® technique using Vydac Everest C_{18} 300 Å 5 μm (250 × 4.6 mm i.d.) column: (1) UV at 215 nm and (2) radioactivity. Peaks at 3.3–4.5, 13.9, 16.1, 17.9, 20.4 and 22.8 min correspond to (a) free iodine, (b) non- (NIP), (c) mono- (MIP) and (d) di- (DIP), (e) tri- (3IP) and (f) tetra-iodinated peptide (4IP), respectively.

iodide added. Clearly, the formation of poly-iodinated obestatin is favoured at higher NaI/peptide ratios. Using our models for the chloramine-T and Iodo-Gen® techniques, pH had a positive effect on MIP yield. This effect was described only for Iodo-Beads®, *i.e.* a solid-state variant of CAT, and was previously explained by the His-pK_a value directing the nitrogen protonation [17]. However, in this case, the phenomenon could not be attributed to an increasing proportion of histidine iodination at higher pH as the percentage of [I_1-His^{19}]-obestatin (calculated with reference to the total amount of MIP) did not exceed 10% for any of the experiments performed. Increasing the pH did thus increase not only His-MIP but Tyr-MIP as well to the same extent. For lactoperoxidase at lower NaI/peptide ratios, a negative pH effect is observed, *i.e.* less mono-iodinated obestatin is formed at higher pH. This is explained by the optimum enzyme activity pH which is stated by the supplier as being around 6.0. The reaction time showed no statistically significant effects and no interactions for the lactoperoxidase technique. A negative effect of reaction time on MIP formation was found for Iodo-Gen®: longer reaction times decreased the MIP formation.

In the synthesis involving chloramine-T, the negative effect on MIP yield of an excess oxidizing agent relative to iodide is related to the positive effect on the formation of di-iodinated obestatin.

The higher iodinated peptides are generally not wanted in radioligand and ADME studies of the native peptide, but they can be useful in comparative and displacement studies. Overall, the most important parameter on the formation of higher iodinated peptides is found to be again the amount of iodide added.

Peptide recovery is an important parameter for efficiency reasons as well as to explain other responses. For the chloramine-T technique, the interaction between reaction time and amount of sodium iodide might be attributed to oxidative degradation when an excess of NaI is used.

In contrast, a significantly higher peptide recovery was obtained when increasing the sodium iodide/peptide ratio from 0.25 to 4 for the lactoperoxidase iodination techniques. This finding is considered to be related to the milder reaction mechanism.

4.2.1. Radio-HPLC analysis of obestatin labelled with ^{125}I using Iodo-Gen®

Formation of higher iodinated obestatin species by non-carrier-added direct radiolabelling was clearly demonstrated for the Iodo-Gen® technique. Being a solid-phase oxidizing agent, Iodo-Gen® was expected to be more receptive for poly-iodination. As these poly-iodinated compounds are expected to present different biological activities and/or pharmacokinetic properties compared to the main compound MIP, it is recommended to verify their absence/presence and remove these compounds if necessary. However, in the radioligand studies reported so far [1,19], neither chemical characterisation, nor isolation of pure mono-iodinated obestatin was performed. Our results demonstrate that a mixture of iodinated obestatin is expected under these synthesis conditions.

5. Conclusions

Optimizations of iodinated obestatin species yields (MIP, DIP, 3IP and 4IP) using Draper-Lin and Box-Behnken designs were performed for three widely used direct labelling techniques: chloramine-T, Iodo-Gen®, and lactoperoxidase. Amount of sodium iodide, pH, CAT/sodium iodide ratio, and reaction time, were taken as factors. The optima of these factors were determined based on the polynomial equations generated by response surface modelling. The results may be applied on both carrier-added and non-carrier-added radioiodinations. The importance of analytical characterization of the iodinated obestatin formed by one of the three reactions is demonstrated. The HPLC separation of [I_1-Tyr^{16}]-obestatin and [I_1-His^{19}]-obestatin, which are formed by all three iodination techniques, is described. Under all conditions tested, the latter compound was found to be less abundant when compared to the obestatin mono-iodinated at Tyr^{16}, with no significant differential influence of the reaction parameters.

Acknowledgments

We acknowledge financial support from the Flemish Institute supporting Scientific-Technological Research in industry (IWT): this work was performed within the SBO-peptidomics project (number 050164). We would like to thank Chris Vervaet (UGent) for the use of the Lyovac.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2007.10.005.

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