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Radiolabeling of A Cyclic RGD (cyclo Arg-Gly-Asp-d-Tyr-Lys) Peptide Using Sodium Hypochlorite As An Oxidizing Agent

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Abstract

A simple and rapid non-radioactive iodide labeling/radiolabeling method for peptides, using an inexpensive oxidizing agent such as sodium hypochlorite and a cyclic peptide, cRGDyK (cyclo Arg-Gly-Asp-d-Tyr-Lys), was developed in this work. Labeling reaction was optimized by conducting experiments under variable ratios of the reagents, the reaction times, and the pH. The study demonstrated that radiolabeling of the cyclic peptide was fast and pH independent. Mono- and di-iodinated cRGDyK were formed under all conditions and varied with the ratio of the reagents and the reaction time. Total percent of the iodinated cRGDyK (mono- and di-iodinated cRGDyK) varied between 44 and 100 depending on the reaction conditions. Excess cyclic peptide over equal molar ratio of sodium iodide and sodium hypochlorite yielded in predominant amounts of mono iodinated cRGDyK, i.e., >60% under 2:1:1 ratio and ~88% under 5:1:1 ratio of cRGDyK: sodium iodide: sodium hypochlorite.

Keywords

Radiotracers; Radiolabeling; Radioiodination; Tyrosine; Iodination; Peptides; Oxidation; Sodium Hypochlorite; Hypochlorous Acid

INTRODUCTION

Radio-tracers have been used for in-vitro and in-vivo evaluations of New Chemical Entities (NCEs) and biomolecules for a long time¹. Radiolabeling of a tyrosine residue in peptides and proteins involves, an iodide radionuclide and an oxidizing agent, including Chloramine-T,² Iodobeads,³ Iodogen,⁴ ICl,⁵ and IBPy2BF₄.⁶ Alternatively, a primary amine in molecules or biomolecules, however, can be indirectly radiolabeled by using Bifunctional Chelating Agents (BFCs) and metallic radionuclides.^{7–12}

The main objective of the present study was to evaluate an oxidizing agent which is easily available and is cost-effective for radiolabeling of tyrosine residue in peptides. Sodium hypochlorite is of particular interest as it oxidizes I[−] to I⁺ or ICl (similar to the existing oxidizing reagents, except IBPy2BF₄) via chlorine atom transfer and the rate of the oxidation reaction is known to be very fast under neutral pH conditions.¹³ Consequently, we

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conducted a systematic $^{127}\text{I}/^{125}\text{I}$ labeling study of a cyclic peptide (cRGDyK, cyclo Arg-Gly-Asp-d-Tyr-Lys) for development of an additional radiolabeling technique. Selection of a cyclic peptide in this study is interesting as (1) several radiolabeled cyclic RGD peptides have been investigated as potential radiotracers for angiogenesis imaging,^{14–22} and (2) Sodium Hypochlorite mediated radiolabeling of peptides and proteins that contain primary amines along with tyrosine residue, have a potential to form N-chloro derivatives,²³ consequently, the selection of a cRGDyK cyclic peptide containing a tyrosine and a lysine residues also provides insight into the selectivity of the non-radioactive labeling/radiolabeling procedure.

The present study demonstrates that sodium hypochlorite can be used successfully for non-radioactive/radio labeling of tyrosine residue in the peptides. The non-radioactive/radiolabeling reaction is rapid and is usually complete in a few minutes. Additionally, the results related to various optimization parameters, such as the ratio of reagents, the reaction times, and the effect of pH on the labeling of the cyclic peptide, cRGDyK, are also presented here.

EXPERIMENTL SECTION

Materials and Methods

A cyclic RGD peptide containing tyrosine residue, cRGDyK (American Peptide), Sodium Iodide (Acros), Sodium Hypochlorite (Fisher), and Sodium Bisulfite (a mixture of NaHSO_3 and $\text{Na}_2\text{S}_2\text{O}_5$, Sigma) were all reagent grade and were used as received. Buffers were prepared and pH and ionic strength were controlled by using Sodium monobasic phosphate sodium dibasic phosphate, sodium hydroxide, hydrochloric acid and sodium chloride, respectively (all from Fisher). Gibco $1\times$ PBS (pH 7.4) buffer was supplied by Life Technologies. Crude reaction mixtures from the non-radioactive labeling/radio labeling reactions were purified, to remove unreacted non-radioactive or radioactive sodium iodide, by using Sep-PaK Light C_{18} cartridges (Waters). For radiolabeling experiments $^{125}\text{I}\text{Na}$ was purchased from Perkin Elmer.

The amounts of radioactivity in the ^{125}I -labeled materials were measured by using a Capintec dose calibrator. For purification and analysis of non-radioactive and radioactive materials Agilent model 1100 HPLC systems controlled by Chem Station software were used. For detection of radio labeled materials a Flow Scintillation Analyzer (FSA 150) from Perkin Elmer or a Flow Ram from Lab Logic Systems was used. A Capintec well counter was used for analysis of paper chromatography samples. A Bruker amazon ETD mass spectrometer at Campus Chemical Instrument Center (CCIC) Mass Spectrometry and Proteomics Facility at The Ohio State University (OSU) was used for ESI mass spectral analysis of samples of non-radioactive iodinated cRGDyK.

In a typical non-radioactive labeling/radiolabeling experiment, known amounts of a cyclic peptide solution in PBS and sodium hypochlorite were added sequentially to a $100\text{ }\mu\text{L}$ of 0.1 M Sodium Phosphate (pH 7.4) buffer solution in a 1.5 mL Eppendorf centrifuge tube followed by addition of the desired amount of sodium iodide or carrier-free $^{125}\text{I}\text{Na}$ activity. Since the $^{125}\text{I}\text{Na}$ sample is supplied in a 0.1 N sodium hydroxide base solution, it was

occasionally necessary to adjust the pH of the solution to 7.4 by the addition of a small amounts of hydrochloric acid. The reaction mixture was agitated and mixed after addition of each reagent by using a pipette and incubated at room temperature for a desired time. At the end of the incubation period, the non-radioactive labeling or radiolabeling reaction was quenched by the addition of a large excess of a reducing agent, sodium bisulfite. Purification of the reaction mixture of the non-radioactive labeled or the radiolabeled cyclic peptide was accomplished in two steps. A reversed-phase Sep-PaK C₁₈ Light cartridge was used to remove any unreacted sodium iodide or ¹²⁵I Na followed by a reversed phase HPLC purification method to remove any unlabeled or unreacted cyclic peptide. Similar procedures were used for non-radioactive labeling/radiolabeling experiments using Iodogen reagent.

For removal of unreacted iodide or ¹²⁵I, a Sep-Pak C₁₈ Light cartridge was conditioned initially by using 3 mL absolute ethanol and 3 mL of water, reaction mixture (~200 µL) was loaded onto the cartridge, washed with 1.0 mL with water, and finally eluted with four to eight 250 µL portions of absolute ethanol. Alternatively, acetonitrile was used in some purification experiments and provided same results. For radiolabeled samples, each fraction was analyzed for unreacted, i.e., free ¹²⁵I Na by using a Paper Chromatography method, involving a Whatman 3 MM cellulose chromatography paper strip and a 9:1 mixture of methanol: water as a developing solution. After the paper was developed and allowed to dry, the strip was then cut into two pieces one-third from the bottom and two third from the top. Each piece of the strip was counted in a well counter.

All ethanol fractions containing iodinated or radio iodinated materials without free iodide or free ¹²⁵I Na were combined and concentrated at 45°C and further purified and analyzed by using a Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) method involving a Zorbax C-18 5 µm, 4.6 × 250 mm column, flow rate of 1 mL/min, and UV detection at λ = 280 nm, a radioisotope detector, and a gradient mobile phase. The following gradient of water containing 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B) was used: 95% A and 5% B initially, ramping the concentration of B to 25% in 20 minutes, ramping down B to 5% in one minute and keeping 5% B for the next 4 minutes.

RESULTS AND DISCUSSION

Non-Radioactive Iodide Labeling of cyclo Arg-Gly-Asp-d-Tyr-Lys (cRGDyK) Using Iodogen Reagent

Initial non-radioactive labeling experiments with cRGDyK were conducted using the Iodogen reagent under variable ratios of cRGDyK and NaI reagents (1 to 100) and incubation times (5 to 60 min). In each case peaks (retention times given in parenthesis) related to unreacted cRGDyK (8.6), mono- (I-cRGDyK) (13.8) and di-iodinated (I₂-RGDyK) (17.1) in variable amounts were observed in the HPLC chromatograms and the identity of these were confirmed by ESI mass spectral analysis.

For example, an HPLC analysis of a 1:1 mixture of cRGDyK (1 µmole) and sodium iodide (1 µmole) incubated in an Iodogen tube for 5 minutes showed, an unreacted cRGDyK (77%), and formation of I-cRGDyK (20%), I₂-cRGDyK (3%). An ESI mass spectral analysis of the three HPLC fractions showed m+H peaks at the following (*m/z*) (calculated

mass in the parenthesis): 620.3 (619.7), 746.2 (745.6), and 872.1 (871.5), respectively. A 1:40 mixture of cRGDyK and sodium iodide incubated for 60 minutes did not improve labeling significantly, however, the ESI MS showed a peak at 780.9 (m/z) suggesting the formation of N-Chloro derivative of mono iodinated cRGDyK (calculated $m+H$ peak at 781.14). Combination of extended incubation time to 60 minutes and the nature of the labeling reagent ($\sim 20 \mu\text{g}$ iodogen reagent coated on the solid surface of the tube) may be responsible for formation of N-Chloro derivative of I-cRGDyK after extended incubation of the reagents.

Non-Radioactive Iodide Labeling of cyclo Arg-Gly-Asp-d-Tyr-Lys (cRGDyK) Using Sodium Hypochlorite

Various parameters (i.e. the ratio of the reagents, the reaction time, and the pH of the reaction) were investigated for development and optimization of an efficient process for non-radioactive labeling of cRGDyK (Table 1). Keeping cRGDyK and sodium iodide in equimolar, but varying the sodium hypochlorite concentration in 20 to 51 fold excess and incubating for 5 minutes, yielded an average ($n=4$) of unreacted cRGDyK, I-cRGDyK, and I_2 -cRGDyK 34%, 48%, and 18%, respectively (Table I and Figure 1). Extended incubation time did not improve the percent formation of I-cRGDyK and I_2 -cRGDyK concluding that a 5 minutes incubation time may be sufficient for non-radioactive and radiolabeling experiments. The percent of I_2 -RGDyK increased significantly, 60 to 80%, when a 1:2 mixture of cRGDyK and sodium iodide was reacted with 2 to 177 fold excess of sodium hypochlorite, suggesting sodium iodide should be a limiting reagent in the non-radioactive labeling reaction as is the case in the carrier free ^{125}I radiolabeling of peptides and proteins. The formation of I-RGDyK and I_2 -RGDyK after one and five minute incubations were essentially same at pH 4.6 and 7.4 suggesting hypochlorous acid being the reactive form of sodium hypochlorite, i.e. protonation constant of OCl^- is $3.1 \times 10^7 \text{ M}^{-1}$).¹³ Longer incubation time, five minutes, appears to produce higher percentage of I_2 -cRGDyK. The non-radioactive labeling experiments conducted in 2:1:1 and 5:1:1 ratios of cRGDyK:NaI:NaOCl at different incubation times demonstrated that the reaction was complete within one minute and forms I-RGDyK predominantly, i.e., 63 and 88%, respectively (Table I).

For characterization of the reaction products a 1:1:50 reaction mixture of cRGDyK:NaI:NaOCl in PBS buffer (pH 7.4) was incubated for five minutes and purified by HPLC. The two peaks at retention times 13.8 and 17.1 minutes were collected, lyophilized, and characterized by ESI MS. The materials collected showed (m/z) at 746.24 vs. calculated 746.21 ($m+H$) for I-c(RGDyK) and (m/z) at 872.10 vs. calculated 872.48 ($M + H^+$) for I_2 -c(RGDyK). No N-Chloro derivative of I-cRGDyK was observed in these labeling experiments, although chlorination of cRGDyK by hypochlorite is not excluded at longer reaction times.

From the data given in this study, it is concluded that the sodium hypochlorite method is inexpensive, rapid, and yields higher percentage of iodinated cRGDyK after five minutes reaction than the Iodogen method. After five minutes of incubation in both methods there was no evidence of formation of a N-Chloro derivative. However, extended incubation of the

reagents in Iodogen method gave N-Chloro derivative which may be possible in the sodium hypochlorite method also.

Radioactive Iodide Labeling of cyclo Arg-Gly-Asp-d-Tyr-Lys (cRGDyK)

The non-radioactive labeling method of cRGDyK was translated into production of ^{125}I labeled cRGDyK. A known amount of the peptide (28 ng, 46 pmol) in PBS (pH7.4) was added to a 1.5 mL polypropylene tube containing phosphate buffer followed by addition of carrier free $^{125}\text{I}\text{Na}$ (~100 μCi) and sodium hypochlorite (170 ng, 46 pmol) in phosphate buffer (pH 7.4). The reaction mixture was agitated and mixed with the pipette and the reaction mixture was incubated at room temperature for 5 min followed by quenching of the reaction with excess sodium bisulfite. Unreacted $^{125}\text{I}\text{NaI}$ was removed from the reaction mixture by using a conditioned Sep-Pak C₁₈ Light cartridge using a method given above. Each ethanol fraction was analyzed for purity by using the paper chromatography method, the R_f values for radiolabeled peptide and iodide were 0.03, and 0.59, respectively and by the HPLC method. Five fractions containing the ^{125}I labeled cRGDyK gave 42% yield of the radiolabeling. First two fractions gave a mixture of ^{125}I -cRGDyK and $^{125}\text{I}_2$ -cRGDyK and later three fractions gave pure ^{125}I -cRGDyK. The fractions containing ^{125}I -cRGDyK were combined and evaporated at 50° C under the stream of nitrogen to remove ethanol. The final residue was reconstituted in PBS and Figure 2 shows a chromatogram of final radiolabeled ^{125}I -cRGDyK.

CONCLUSIONS

A rapid radiolabeling method for molecules and biomolecules containing tyrosine residue has been developed and optimized. The study demonstrated that the radiolabeling of the cyclic peptide is fast (i.e., less than five minutes are sufficient) and the pH independent. Mono- and di-iodinated cRGDyK formed under all conditions and varied with the ratio of the reagents. Total percent of the iodinated cRGDyK (mono- and di-iodinated cRGDyK) varied between 44 to 100% depending on the reaction conditions. Excess cyclic peptide over equal molar ratio of sodium iodide and sodium hypochlorite yielded in predominant amounts of mono iodinated cRGDyK, i.e., >60% under 2:1:1 ratio of cRGDyK: sodium iodide: sodium hypochlorite and >88% under 5:1:1 ratio of cRGDyK: sodium iodide: sodium hypochlorite.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Representative Reversed-Phase HPLC Chromatogram Post Non-Radioactive Labeling of
cRGDyK (cyclo Arg-Gly-Asp-d-Tyr-Lys).

Figure 2.
Representative Reversed-Phase HPLC Chromatogram of ¹²⁵I-c(RGDyK).

Table 1

Percent Yield of Non-Radioactive Labeling of cyclo Arg-Gly-Asp-d-Tyr-Lys (cRGDyK) Under Various Conditions, the pH, the Molar Ratios of Reagents, and the Reaction Times.

pH	cRGDyK	NaI	NaOCl	Time, Min	% cRGDyK	% I-cRDyK	% I ₂ -cRGDyK
7.4	1	1	20	5	39.2	46.0	14.8
7.4	1	1.2	22	5	40.0	45.3	14.7
7.4	1	1	51	5	26.2	51.3	22.5
7.4	1	1	50	5	30.6	49.7	19.7
7.4	1	1.2	22	5	40.0	45.3	14.7
7.4	1	1.2	22	10	41.8	44.6	13.6
7.4	1	1.2	22	15	41.8	40.8	12.3
7.4	1	1.2	22	20	43.4	43.7	12.9
7.4	1	2	89	5	4.4	12.3	83.3
7.4	1	2	177	5	5.2	13.7	81.1
7.4	1	2	2	0.5	10.4	29.0	60.6
7.4	1	2	2	1	10.6	29.9	59.5
7.4	1	2	2	2	10.5	30.5	59.0
7.4	1	2	2	3	10.5	30.6	58.9
7.4	1	2	2	4	11.1	30.6	58.3
7.4	1	1	20	1	56.5	37.7	5.8
7.4	1	1	20	5	39.2	46.0	14.8
4.6	1	1	20	1	50.4	41.2	8.4
4.6	1	1	20	5	36.1	47.1	16.8
7.4	2	1	1	1	13.6	64.4	22.0
7.4	2	1	1	2	14.2	64.2	21.6
7.4	2	1	1	3	13.3	68.7	18.0
7.4	2	1	1	4	13.5	63.1	23.3
7.4	5	1	1	1	0.0	87.6	12.4
7.4	5	1	1	5	0.0	87.9	12.1