New Advances towards Quantitative Proteomics: Optimization of Peptide Labeling by Fluorescent Isotope Coded Affinity Tag (FCAT)

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Introduction

One of the most important subjects in contemporary proteomics is the relative and absolute quantification of gene expression at the protein level. The application of stable-isotope labeling in mass spectrometry for the analysis of proteins and peptides has proven successful to reveal comparative proteome-wide changes in gene expression at the protein level (*1*). Recent efforts in proteomics are focused towards global studies requiring quantitative and comparative protein analysis. Since the introduction of the first version of isotope-coded affinity tag (ICAT) reagent in 1999 *(2),* similar reagents have been widely applied in quantitative proteomics (*3*). We have reported earlier on the design and synthesis of a novel fluorescent isotope-coded affinity tag (FCAT) to provide an additional tool for quantitative proteomics. The design of the FCAT 1 and FCAT 2 molecules are shown in Figure 1. The molecular structures contain some similar motifs. Section A in Figure 1 depicts the fluorescein residue that supports quantification by fluorescent LC detection and also serves as affinity tag

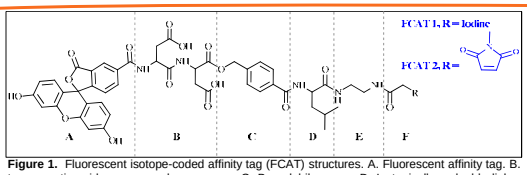

Figure 1. Fluorescent isotope-coded affinity tag (FCAT) structures. A. Fluorescent affinity tag. B. two aspartic acid groups used as spacers. C. Base labile group. D. Isotopically codeable linker (leucine for the light form and leucine ^{13}C and ^{15}N for the heavy form). E. ethylendiamine residue used as spacer and F. Thiol-specific reactive group.

to selectively isolate the FCAT labeled peptides by immunoprecipitation using a corresponding antibody. Section C in Figure 1 shows the 4-hydroxymethylbenzoic acid (HMBA) group that was included into the structures to provide a cleavage site for the fluorophore group of the reagent by a simple base treatment before mass spectrometry. Section D in Figure 1 is the stable isotope carrying part. Leucine was chosen since its availability with stable ^{13}C and ^{15}N labeled forms. Section F in Figure 1 shows the reactive groups of iodoacetyl (FCAT 1) or maleimide (FCAT 2) towards the sulfhydryl groups of cysteine containing peptides and proteins. Aspartic acids and ethylendiamine residues were used as spacers (Section B and E in Figure1).

The use of the FCAT reagent in proteomics is illustrated in Figure 2. First, the protein samples of interest are reduced in order to generate free thiol groups. Then one sample is labeled with the light FCAT reagent, the other one is with the heavy FCAT reagent. The two samples are then combined and digested to obtain a mixture peptides of which the cysteine containing ones are labeled with the heavy or light FCAT reagents. The FCAT labeled peptides are then subject to affinity chromatography using a fluorescein motif recognizing antibody to partition the labeled peptides only. This step is followed by high resolution HPLC analysis with fluorescent detection for absolute quantification. Finally, the eluent of the HPLC column is sodium hydroxide treated to cleave off the fluorescent motif and the resulting peptide pairs are analyzed by LC-MS to determine their relative abundance. After the carefully planned design of the FCAT reagents, we successfully synthesized these molecules. In this poster we describe the testing of the new labeling agent with iodoacetyl and maleimide reactive groups using model cysteine containing peptides.

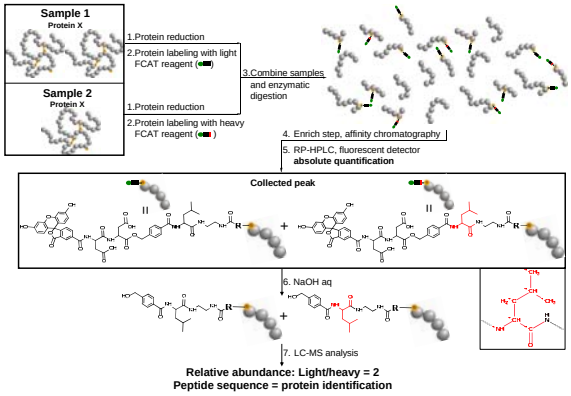

Figure 2. Use of Fluorescent Isotope Coded Affinity Tag (FCAT) reagents in quantitative proteomics

Methods

Evaluation of FCAT reactivity. *Peptide Labeling with FCAT Reagents.* Two cysteine containing peptides were selected to check the reactivity of the synthesized FCAT reagents: Peptide 1: Cys-Ala-Ser-Ile-Gln-Lys-NH_2 (**C**ASIQK-NH_2), and Peptide 2: Asn-Cys-Gln-Phe-Glu-Lys-OH (N**C**QFEK-OH) from FIDIC (Bogotá,Colombia). 10 mM solutions were prepared in water. 20 nmol of each peptide were reduced by adding 9 µL of 6.7 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and incubating for 30 min at 25°C. TCEP solution was prepared in 50 mM TRIS-HCl buffer (pH 8.48) containing 0.1 % SDS (w/v). The final pH was pH 7.40.

Labeling reaction with FCAT 1 (reactive group: iodoacetyl). 10 nmol of each peptides were reduced and treated with 40 nmol of FCAT 1 in TRIS-HCl buffer pH 8.48 (4 mM final concentration). The reaction mixtures were incubated for 4 h at 37°C, protected from light and kept overnight at 4°C. Then, 10 µL of 0.1 M DTT was added and incubated for 30 minutes at room temperature. Finally the solutions were diluted 10 times with 10 % acetonitrile in water and desalted using PhyTip Polymer Reversed Phase pipette tips (PhyNexus, San Jose, CA, USA) using 50% acetonitrile elution buffer.

Labeling reaction with FCAT 2 (reactive group: maleimide). 10 nmol of each reduced peptide was labeled with 40 nmol of FCAT 2 in 50 mM TRIS-HCl buffer (pH 7.01) containing 0.1 % SDS (w/v) and incubated for 4 hours at 37°C, protected from light. The reaction mixtures were kept overnight at 4°C and the reactions were then stopped by the addition of 0.1 M DTT and incubated for 30 minutes at room temperature. Finally, each solution was diluted 10 times with 10% acetonitrile in water and desalted using PhyTip Polymer Reversed Phase pipette tips using 50% acetonitrile elution buffer. The labeling efficiency was monitored by MALDI-TOF MS and LC-MS.

Cleavage of the Fluorophore group. The FCAT reagent labeled model peptides were treated with sodium hydroxide (final concentration 0.15 M) for 15 minutes at room temperature to cleave off the fluorophore part of the molecule. After desalting by PhyTip Polymer Reversed Phase pipette tips (PhyNexus) the efficiency of the cleavage step was checked by MALDI-TOF MS.

Results

Peptide Labeling with FCAT Reagents. The completeness of the FCAT 1, 2 reactions with cysteine containing peptides was analyzed by MALDI-TOF MS and RP-HPLC. Table 1 summarizes all peaks/signals presents in MALDI-TOF MS spectra for FCAT 1 and 2 with the two Cys containing peptides and the control peptide. In addition to the characteristic signals for the labeled peptides, peaks for FCAT conjugated reducing agents were also found (i.e., with dithiotreitol, DTT and tris(2-carboxyethyl)phosphine, TCEP). A control reaction was also performed with a peptide having no cysteine residue. In this later instance, we only observed peaks corresponding to labeled DTT, suggesting the high specificity of the FCAT reagent towards thiol groups. Products of labeling reactions were purified by RP-HPLC and analyzed by LC-MS-MS. It was possible to call the complete sequence of the labeled peptides 1 and 2. An example shown for peptide 1 in Figure 3. In addition, the MS-MS analysis of side product corresponding to TCEP reaction with FCAT reagents showed that TCEP is reacting with iodoacetyl and maleimide groups, but no reaction with other part of the FCAT structures was found. Figure 4 shows the MS-MS spectra of this side reactions.

Label / Sample	Labeled products						Without fluorescent motif			
	Theoretical MW_{mi}			Experimental $[M+H]^+$			Theoretical MW_{mi}		Experimental $[M+H]^+$	
	-	FCAT 1	FCAT 2		FCAT 1	FCAT 2	FCAT 1	FCAT 2	FCAT 1	FCAT 2
	-	1063.20	1046.32	-	1064.02	1047.16	-	-	-	-
Peptide 1: CASIQK	647.34[1] 1292.68[2]	1582.63	1693.66	648.07 1293.81	**1583.76** 1090.36[a1] 2025.66[a2] 1186.44[b]	**1694.62** 1201.33[a1] 2247.33[a2]	994.53	1105.56	**995.56**	**1124.69**
Peptide 2: NCQFEK	767.33[1] 1532.64[2]	1702.61	1813.65	768.24 1533.12	**1703.78** 1090.42[a1] 2026.73[a2] 1186.51[b]	**1814.90** 1201.56[a1] 2247.70[a2] 1297.64[b]	1114.51	1225.54	**1115.59**	**1244.73**
Control peptide SATPASAPYPLAGGGS	1401.69	-	-	1402.83	**1402.83** 1090.32[a1]	**1402.79** 1201.37[a1]	-	-	-	-

1. Monomer (peptides).
2. Dimmer (peptides)
a. Products of reaction between DTT and FCATs; a1 and a2: reaction with one or two molecules of FCAT respectively.
b. Products of reaction between TCEP and FCATs.

FCAT 1: Theoretical MW_{mi} a1: 1089.30; a2: 2024.58; b: 1186.35
FCAT 2: Theoretical MW_{mi} a1: 1200.33; a2: 2246.65; b: 1297.39

Table 1. Reaction between FCAT 1 and FCAT 2 with model peptides. Summary of MALDI-TOF MS analysis

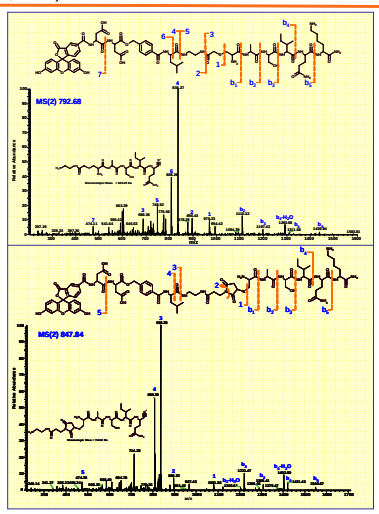

Figure 3. MS-MS spectra of Peptide 1 labeled with FCAT 1 (top) and FCAT 2 (bottom).

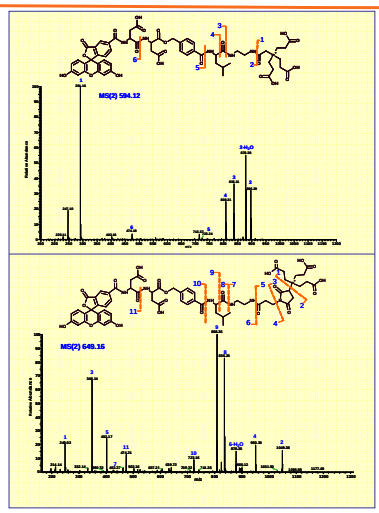

Figure 4. MS-MS spectra of the products from the reaction between TCEP and FCAT 1 (top) and FCAT 2 (bottom), respectively.

Cleavage of the Fluorophore group.

Analysis of the reaction products of FCAT 1 labeled peptides by MALDI-TOF shows the cleavage of the fluorophore group (Table 1). MS-MS analysis depicted the possibility of determining the peptide sequence. On the other hand, products cleavage of the fluorophore group from FCAT 2 labeled peptides showed unexpected peaks with 18 Da higher m/z, while the expected peaks were not found. MS-MS analysis of this product suggested water addition, probably by opening the succinimide ring (hydrolysis). Despite this unexpected reaction, MS-MS analysis of these products proved the option to sequence these peptides.

Conclusion

A novel fluorescent isotope-coded affinity tag (FCAT) was proposed to provide an additional new tool for quantitative proteomics. The tag showed good reactivity with both terminal and internal cysteine groups in model peptides. Labeling reactions were followed by HPLC, MALDI-TOF and chipLC-ESI-MS/MS techniques. The tagging reaction was found to be very efficient. The labeled peptides were sequenced by MS/MS analysis. Basic treatment of the cysteine labeled peptides effectively cleaved off the large fluorophore group of the tag. The labeling reaction was also optimized to avoid nonspecific reactions.

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(1) Julka, S., and Regnier, F. (2004) Quantification in proteomics through stable isotope coding: A review. Journal of Proteome Research 3, 350-363.
(2) Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nature Biotechnology 17, 994-999.
(3) Linscheid, M. W. (2005) Quantitative proteomics. Analytical and Bioanalytical Chemistry 381, 64-66.