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An Improved Isotope Coded Affinity Tag Technology for Thiol Redox Proteomics

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ABSTRACT

Isotope Coded Affinity Tag (ICAT) is a gel-free technology for quantitative proteomics. In ICAT procedure, strong cation exchange chromatography (SCX) using increased potassium chloride gradient is recommended for peptide fractionation. Here we report optimization of hydrophilic interaction chromatography (HILIC) as an alternative strategy for peptide fractionation of ICAT samples. HILIC exhibits high separation efficiency and does not require any downstream desalting steps. Compared to SCX based ICAT, integration of HILIC into the ICAT technology has resulted in high rates of protein identification, cysteine mapping, and quantification of cysteine-containing peptides. The improved technology has shown utility in thiol redox proteomics. Interestingly, results from HILIC ICAT and SCX ICAT are complementary. Implementation of both provides high coverage analysis of a complex proteome.

Keywords: ICAT; HILIC; SCX; Proteomics; Mass spectrometry.

1. Introduction

Although two-dimensional gel electrophoresis (2-DE) has been used for decades to separate and quantify proteins on a large scale, its limitations in reproducibility and separation of membrane proteins, acidic, basic, very small and large proteins have promoted the invention of a gel-free isotope-coded affinity tag (ICAT) technology in 1999 [1, 2]. The ICAT reagent consists of a cysteine-reactive group, a linker containing stable isotope signatures and a biotin affinity tag enabling the isolation of cysteine-containing peptides. In the original ICAT reagent, the linker region of heavy form contains eight deuteriums and the light form contains no deuteriums. It was reported later that the light and heavy ICAT tagged peptides exhibited different retention on reverse phase HPLC columns [3]. In addition, the retention of the biotin group complicates tandem mass spectrometry (MS/MS) spectrum interpretation. To overcome the problems, a cleavable ICAT reagent (cICAT) was developed. Carbon-13 was used instead of deuterium in the linker and the mass difference between the heavy and light forms is 9 Dalton. Additionally, an acid cleavable moiety was introduced between the biotin group and the rest of the molecule [4, 5]. Because the ICAT technology overcomes the 2-DE limitations, it has been applied as an alternative to address a variety of biological questions including whole-cell protein expression changes [6-8], protein subcellular localization [9, 10], dynamics of protein complexes [11-13], and identification of redox sensitive proteins [14].

In the ICAT method, two protein samples are labeled with isotopically light and heavy ICAT reagents respectively, which covalently attach to cysteine residues of the samples. The two samples are combined, proteolyzed with trypsin, and the peptides are fractionated by strong cation exchange (SCX) chromatography using potassium chloride gradient. After desalting, ICAT labeled peptides are purified using

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avidin affinity chromatography, followed by acid cleavage of the biotin group. The purified peptides are then subjected to mass spectrometry (MS) analysis for relative quantification and peptide sequencing [4, 7]. Obviously, the ICAT method has the advantage of simplifying sample complexity using SCX fractionation and biotin affinity purification of cysteine-containing peptides. However, SCX separation is solely based on the peptide charge state and often limited by poor retention of similarly charged peptides. Recent studies have shown that hydrophilic interaction chromatography (HILIC) offers a superior separation mechanism that is based on retention by hydrophilicity and electrostatic interaction [15, 16]. The stationary phase of HILIC column (e.g., Luna® HILIC column, Phenomenex Inc., USA) has a silica surface covered with cross-linked diol groups, which has been reported to have broad range of applications with high recovery and reproducibility [17]. Direct comparison of SCX and HILIC in peptide separation has demonstrated the enhanced separation power of HILIC [15, 16, 18, 19]. Since HILIC is a type of normal phase liquid chromatography, volatile solvent can be used and desalting steps for downstream applications are not necessary. HILIC may provide an excellent alternative to SCX in ICAT peptide fractionation.

Since the development of isobaric tag for relative and absolute quantification (iTRAQ) in 2004 [20], it has replaced ICAT in many applications because of the high efficiency of iTRAQ multiplexing and the labeling of all peptides [20-22]. One application that retains the strength of the ICAT technology is thiol redox protein analysis [14, 23]. Here we investigated the use of HILIC to replace SCX recommended in current ICAT method with the focus on the identification of redox sensitive proteins. Control and abscisic acid (ABA) treated guard cell proteins were used as starting materials. After ICAT labeling and trypsin digestion, optimized HILIC conditions were used to fractionate the peptides. The fractions were loaded onto avidin columns without desalting. In parallel, regular SCX with potassium chloride gradient was carried out for comparison. HILIC showed better retention and recovery of ICAT labeled peptides. MS analysis revealed more protein identification and cysteine mapping data of the HILIC samples than the SCX samples. Interestingly, the two methods turned out to be complementary. The improved HILIC ICAT together with the SCX ICAT can provide greater in-depth coverage analysis of quantitative changes of reactive cysteines in complex protein samples.

2. Material and Methods

2.1 *Plant growth, guard cell isolation and ABA treatment.*

Brassica napus var. Global seeds were kindly provided by Svalöv Weibull AB (Svalöv, Sweden). Plant growth and guard cell isolation were carried out as previously described [21]. ABA was incubated with the guard cells at 100 μM for 3 hours during the isolation [22].

2.2 *Protein preparation and ICAT labeling.*

A solution of 10% trichloroacetic acid (TCA) in acetone was used to precipitate protein on ice for 2 hours. Samples were washed with 80% acetone once followed by washing with 100% acetone twice. Pellets were dissolved in the ReadyPrep™ Sequential Extraction Reagent 3 (Bio-Rad Inc., USA) and quantified by a CB-X™ protein assay kit (G Biosciences Inc., USA). Protein aliquots of 100 μg were alkylated with 100 mM iodoacetamide (IAM) at 75°C for 5 min, followed by 1 hour incubation at 37°C [24]. Protein samples were precipitated in 100% cold acetone over night. The pellets were dissolved in 80 μL denaturing buffer (pH 8.5) provided in the ICAT kit (AB Sciex Inc., USA). Reduction, ICAT labeling and trypsin digestion were performed according to the manufacturer's manual. The tryptic peptides were fractionated using an Agilent 1100 HPLC with a Luna® HILIC column (150 × 2.0 mm, 3 μm, 200 Å, Phenomenex Inc., USA) or with a PolySULFOETHYL A™ SCX column (150 × 2.1 mm, 5 μm, 300 Å, Poly LC Inc., USA). Mobile phases for HILIC were 5 mM ammonium acetate in 90% acetonitrile, pH 5.8 as solvent A and 5 mM ammonium acetate in water, pH 5.8 as solvent B. Peptides were eluted at a flow rate of 200 μL/min with a linear gradient of 0-50% solvent B over 50 min, followed by ramping up to 100% solvent B in 5 min and holding for 5 min before equilibrating in 0% solvent B. Mobile phases for SCX were 10 mM KH_2PO_4, 25% acetonitrile, pH 3 as solvent A and 10 mM KH_2PO_4, 350 mM KCl, 25% acetonitrile, pH 3 as solvent B. Peptides were eluted at a flow rate of 200 μL/min with a linear gradient of 0-100% solvent B over 60 min, followed by holding at 100% solvent B for 5 min before equilibrating in 100% solvent A. The absorbance at 214 nm was monitored and a total of 10 fractions were collected. The SCX fractions were desalted by solid phase extraction (SPE) using Vydac® silica C18 MacroSpin™ column (The Nest Group Inc., USA). The peptides from each fraction were affinity purified using an avidin affinity cartridge provided in the ICAT kit. ICAT labeled peptides were released by incubating with the cleavage reagent at 37°C for two hours, followed by lyophilization to dryness (Figure 1).

2.3 *Protein identification using LC-MS/MS, database searching and data analysis.*

ICAT labeled peptides were dissolved in 10 μL solvent A (0.1% v/v acetic acid, 3% v/v acetonitrile) and loaded onto a C18 PepMap™ nanoflow column (75 μm id, 3 μm, 100 Å, Dionex, USA). The elution gradient of the column started at 3% solvent A, 97% solvent B and finished at 60% solvent A, 40% solvent B for 60 min. Solvent A consisted of 0.1% v/v acetic acid, 3% v/v acetonitrile, and 96.9% v/v water. Solvent B consisted of 0.1% v/v acetic acid, 96.9% v/v acetonitrile, and 3% v/water. Tandem MS analysis was carried out on a

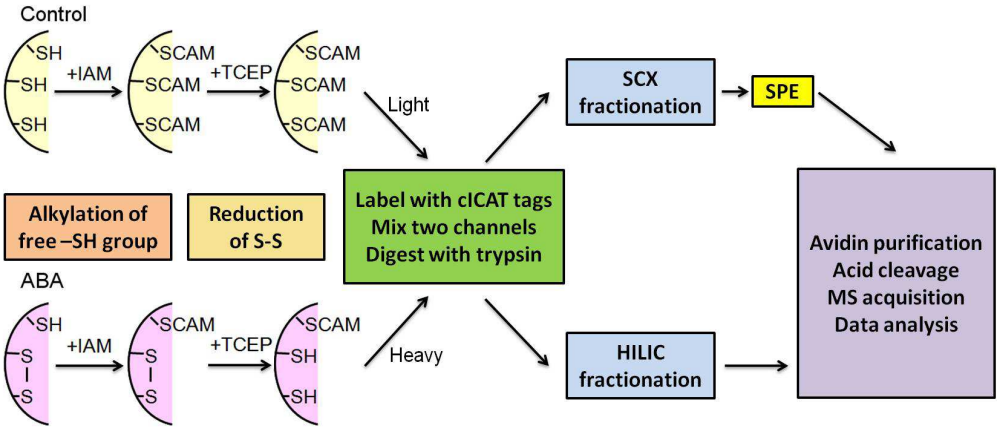

Figure 1. ICAT workflow with SCX and HILIC for thiol-based redox sensitive protein identification.

hybrid quadrupole time-of-flight mass spectrometer (QSTAR® XL, AB Sciex Inc., USA) as previously described [24]. Proteins were identified by searching the MS/MS data against a custom database containing *Arabidopsis thaliana* and *B. napus* protein sequences (downloaded from NCBI with a total of 33,365 entries) using ProteinPilot™ 4.0 software (AB Sciex Inc., USA) because the complete *B. napus* genome is not available and *A. thaliana* is a close relative sharing up to 87% protein sequence identity [25]. The following criteria were used to identify redox sensitive cysteines and proteins: i) at least 20% change of ICAT peptide ion intensity under ABA treatment [14] (Supplemental Figure 1), ii) peptide confidence over 95%, iii) peptide present in at least two replicates, and iv) each peptide assigned to only one protein. The MS data reported in this paper are available in the PRIDE database (www.ebi.ac.uk/prid) [26, 27] under accession number 16864-16867.

3. Results and Discussion

3.1 *Optimization of HILIC conditions for peptide fractionation.*

The factors that affect HILIC chromatography include type and content of organic solvent, salt concentration and pH, in addition to stationary phase properties. A HILIC buffer typically contains more than 70% acetonitrile and uses ammonium acetate or formate [15, 19, 28, 29]. This is because these reagents are volatile and compatible with mass spectrometry. In addition, among the organic solvents tested, acetonitrile was found to exhibit superior chromatography and analyte retention [29]. Salt was shown to increase the hydrophilicity of the liquid layer around the stationary phase and to facilitate analyte retention [18, 30]. Here we focus on optimizing a critical factor of HILIC chromatography, the pH, using bovine serum albumin (BSA) tryptic digest (100 pmol on column). As shown in Figure 2, it is clear that pH 5.8 (Figure 2A) gave much better retention and resolution of the peptides than pH 3.2 (Figure 2B) and pH 6.8 (Figure 2C). The buffer pH directly affects the charge state and hydrophilicity of peptides, and thus the interaction with the HILIC stationary phase. Under pH 3.2 and 6.8 conditions, the peptide hydrophilicity and electrostatic interaction with the stationary phase were not optimal for retention and separation on HILIC. Therefore, pH 5.8 solvents were chosen for HILIC separation of complex protein digests. Although pH 5.8 gave satisfactory results, it should be noted that the pH conditions can be further optimized by testing more pH units. An optimal pH depends on the pKa values of the peptides and should enable favorable retention and fractionation of most, if not all of the peptides in a sample.

3.2 *ICAT peptide fractionation by HILIC and SCX chromatography.*

Currently, many on-line and off-line 2D-LC MS experiments utilize SCX with reverse phase chromatography [22, 30-32]. In standard ICAT protocol, SCX is a critical step before avidin affinity purification of cysteine containing peptides from complex samples (Figure 1). Recent studies have shown that HILIC offers a superior separation mechanism based on retention by hydrophilicity and electrostatic interaction [15, 16, 18, 19]. Here we conducted a direct comparison of SCX and HILIC in the fractionation of peptides derived from guard cells ICAT samples. Control and ABA treated guard cell proteins were combined, digested with trypsin and the resulting peptides were aliquoted for SCX and HILIC separation. As shown in Figure 2, HILIC (Figure 2D) resembled SCX (Figure 2E), but showed enhanced peptide retention and higher peak intensity compared to SCX. Based on the LC chromatograms, the two separation methods exhibited high reproducibility in an independent experiment (Supplemental Figure 2). Since volatile solvent was used in HILIC, the fractions collected can be lyophilized and used directly for further separation, e.g., reverse phase chromatography without the desalting step that could lead to peptide loss (Figure 1). It becomes evident that HILIC provides an

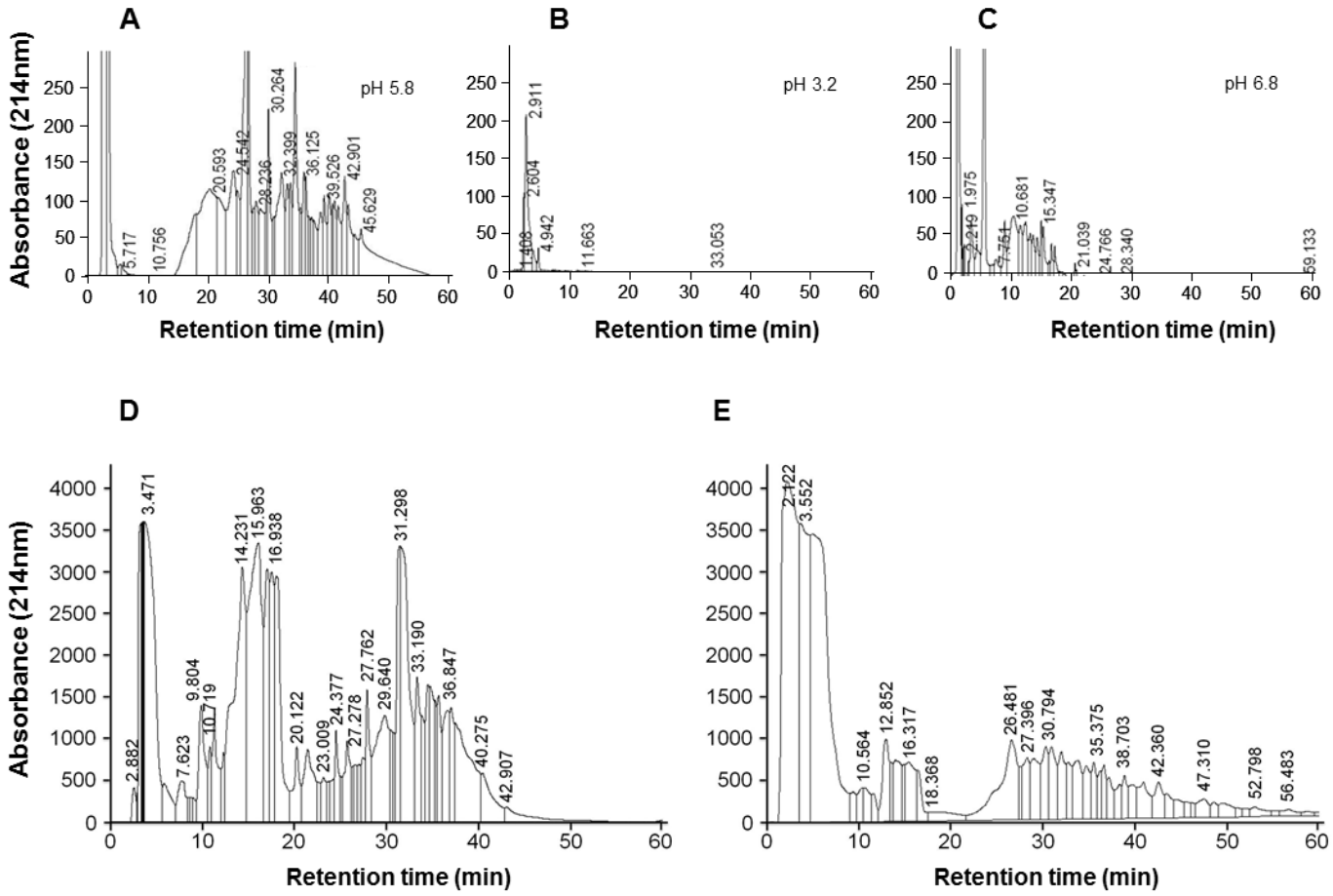

Figure 2. Representative HILIC and SCX chromatograms. (A-C) HILIC chromatograms of 100 pmol BSA digest separated using solvents of different pH values. (D) HILIC chromatogram of guard cell protein digest. (E) SCX chromatogram of guard cell protein digest.

excellent alternative to SCX in peptide fractionation of ICAT experiments.

3.3 *Protein identification and quantification using HILIC ICAT and SCX ICAT.*

In ICAT experiments, proteins can be identified and relatively quantified between two different samples. In addition, cysteine residues labeled by ICAT reagents can be mapped using the acquired MS/MS spectra (Figure 3). To obtain confident results, two independent experiments were conducted. Each experiment included a HILIC ICAT and a SCX ICAT of two guard cell samples, one control and the other ABA treated. It is interesting to note that in the two replicates of SCX ICAT, 59 and 53 proteins were identified, with only 16 proteins overlapping between the two replicates (Figure 4, Supplemental Table 1). In the two replicates of HILIC ICAT experiments, significantly more proteins, 91 and 77 were identified, with over a half of proteins (54 IDs) overlapping (Figure 4, Supplemental Table 1). These results showed the advantage of conducting replicate experiments [33] and the high reproducibility of the HILIC ICAT workflow. Comparison of both HILIC ICAT and SCX ICAT methods revealed that they are highly complementary. HILIC ICAT and SCX ICAT identified a total of 114 and 96 proteins, respectively, with 41 overlapping and the rest unique to each method. In addition to protein identification and quantification, many ICAT labeled cysteine residues were mapped (Figures 4, Supplemental Table 2). Here we analyze redox responsive cysteines after ABA treatment. Among the 44 peptides containing redox sensitive cysteines, HILIC ICAT identified 40 and SCX ICAT identified 30 peptides. Overall, the changes in ICAT ratios were consistent across replicates (Supplemental Table 2). The two methods produced overlapping qualitative as well as quantitative results (Figure 4, Supplemental Table 2). It is interesting to note that in the two replicates of SCX ICAT, only half of the 30 peptides were reproducibly identified. In the HILIC ICAT experiments, more peptides (38 out of 40) were identified in the both replicates (Supplemental Table 2). These results indicate a higher reproducibility of the HILIC ICAT workflow. Overall, the cysteine specificity of the ICAT technology is an inherent advantage for experiments focused on investigating cysteine modifications [14]. However, it

Figure 3. Example of protein identification and quantification result including summary of the protein (A), peptide quantification in the control sample (light) and ABA treated sample (heavy) (B), and peptide MS/MS spectrum indicating heavy ICAT labeled cysteine (C).

may compromise overall protein identification and quantification because non-cysteine containing peptides were excluded from downstream MS analysis. Other technologies such as iTRAQ [21, 22] are an alternative to overcome this limitation.

4. Conclusion

HILIC conditions have been optimized and successfully incorporated into the popular ICAT workflow to replace SCX. Considering the LC chromatograms and the number of proteins characterized, HILIC ICAT has clearly exhibited superior performance to SCX ICAT. The improvement can be attributed to HILIC separation based on retention by hydrophilicity and electrostatic interaction, and the unnecessary desalting steps. Interestingly, for either SCX ICAT or HILIC ICAT, replicate experiments increased the number of proteins identified and cysteines mapped. In addition to some common proteins detected by both SCX and HILIC ICAT methods, each identified a unique set of

A

Experiment	Proteins	Cysteine peptides	Cysteines mapped	Redox peptides
SCX01	59	114	117	21
SCX02	53	141	181	24
HILIC01	91	386	451	40
HILIC02	77	206	228	39

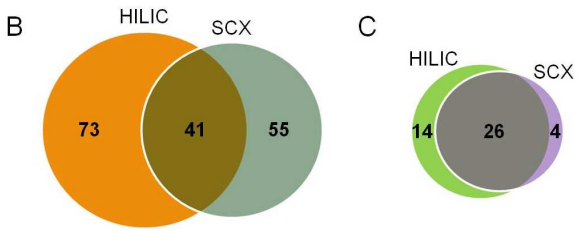

Figure 4. Comparison of protein identification and cysteine mapping results using the SCX ICAT and HILIC ICAT methods. (A) Summary of SCX ICAT and HILIC ICAT results. (B) Proteins identified by HILIC ICAT and SCX ICAT. (C) Significant redox sensitive cysteine peptides quantified by HILIC ICAT and SCX ICAT. (Detailed results are listed in Supplemental Tables 1 and 2).

proteins. Therefore, the novel combination of HILIC ICAT with SCX ICAT can significantly enhance the qualitative and quantitative analysis of proteins, especially thiol redox proteins. The usefulness of HILIC has also been found in shotgun proteomics and phosphoproteomics [16, 19] and can be extended to complex sample fractionation for iTRAQ analysis and the newly released six-plex cysteine reactive TMT tags (Thermo Scientific Inc., USA) in due course.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/83/0

Supplemental Table 1 - List of proteins and peptides identified in *Brassica napus* guard cells using SCX ICAT and HILIC ICAT methods;

Supplemental Table 2 - Redox-sensitive proteins and peptides in ABA treated guard cells identified by SCX ICAT and HILIC ICAT. Increased ratios are highlighted in red and decreased ratios in green. Unused score (for protein) is the summed score for peptides that are not claimed by another protein; the % Error represents the error in the calculated ratio, calculated from the error for each of the peaks in the ratio; R in the redox switch stands for reduction while O stands for oxidation;

Supplemental Figure 1 - Histogram of ICAT ratio distribution of each experiment. (A) SCX-ICAT replicate 1, with average ICAT ratio of 0.87 and % Error 5.48%; (B) SCX-ICAT replicate 2, with average ICAT ratio of 1.08 and % Error 4.42%; (C) HILIC-ICAT replicate 1, with average ICAT ratio of 1.19 and % Error 3.94%; (D) HILIC-ICAT replicate 2, with average ICAT ratio of 1.04 and % Error 4.19%. In all four replicates, the average ICAT ratio is 1.09 with % Error 4.24%.

Supplemental Figure 2 - HILIC chromatogram (left) and SCX chromatogram (right) of guard cell protein digest after labeling the control sample with ICAT light reagent and ABA treated sample with ICAT heavy reagent. The result was obtained from an experiment independent of that shown in Figure 2D and 2E.

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