

Two-Dimensional Analysis of Isotope Coded Affinity Tag (ICAT) Labeled Proteins

INTRODUCTION

Isotope-coded affinity tags (ICAT) reagents are emerging as a powerful tool for quantitative proteome analysis. These reagents, as well as chromatography and tandem mass spectrometry allow the accurate quantitation and identification of proteins in complex mixtures. One distinguishable feature of the ICAT method is the applicability to extremely complex peptides mixtures by reducing the complexity of the samples. This simplification is enabled by biotinylated tags of the reagents by which the ICAT-labeled peptides are selectively retained on an avidin column and then purified. Stable isotopes are bound to cysteine residues by selective alkylation with either a light (D0) or heavy (D8) ICAT reagent. Therefore, the affinity tags result in a mass difference of 8 Da between the two samples and the peptides are easily distinguished using mass spec-

trometry. Indeed, the D8 reagent with 8 deuterium atoms, is 8 Da heavier than the D0 reagent and a comparison of peptides labeled with the two different ICAT reagents provides a ratio of proteins concentration in the original samples (Figure 1).

CONDITIONS

- Nano LC: UltiMate™ / Switchos™ (LC Packings)
- SCX Column: 300 μm I.D. x 5 mm, packed with Spherisorb, 5 μm (LC Packings)
- Loading: 0.1% AcOH in H₂O at 20 μL / min
- Elution Steps: 0-250 mM KCl in 5 mM KH₂PO₄ buffer (pH = 3) with 5 % MeCN
- C18 Column: 75 μm I.D. x 15 cm, packed with Vydac C18, 5 μm (LC Packings)
- Flow Rate: 250 nL / min
- Mobile Phase: A) 0.1% AcOH in H₂O
B) 0.1% AcOH in MeCN / H₂O (95:5, v/v)
- Gradient: 0–100 % B in 30 min
- Injection: 2 μL (FAMOS, LC Packings)
- Detection: UV 214 nm and Mariner-Tof MS (Applied Biosystems)
- Sample: 6 proteins mixture, ICAT labeled & trypsinized, ca. 2 pmol injected

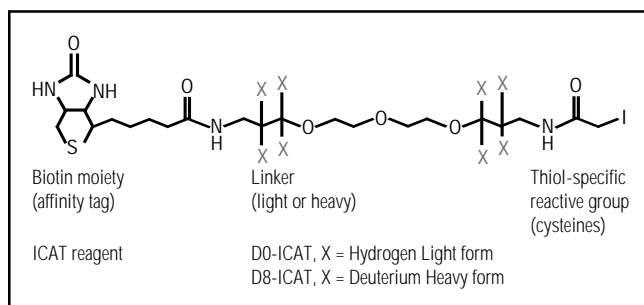


Figure 1. ICAT reagent.

RESULTS AND DISCUSSION

Figure 2 shows the ICAT strategy where proteins isolated from a healthy cell are treated with the light reagent (control sample) while proteins isolated from a diseased cell are labeled with the heavy reagent (A). The two samples corresponding to the two different cell states are then mixed and digested with trypsin. The ICAT-labeled peptides are then separated from the other

peptides by affinity chromatography using an avidin column (B). Indeed, the biotin group on the ICAT reagent possesses a very strong affinity for the avidin stationary phase. After elution of the bound peptides from the affinity media, a separation by miniaturized LC coupled to MS/MS capabilities allows both the quantitation (C) and the identification of proteins (D) contained in the original samples.

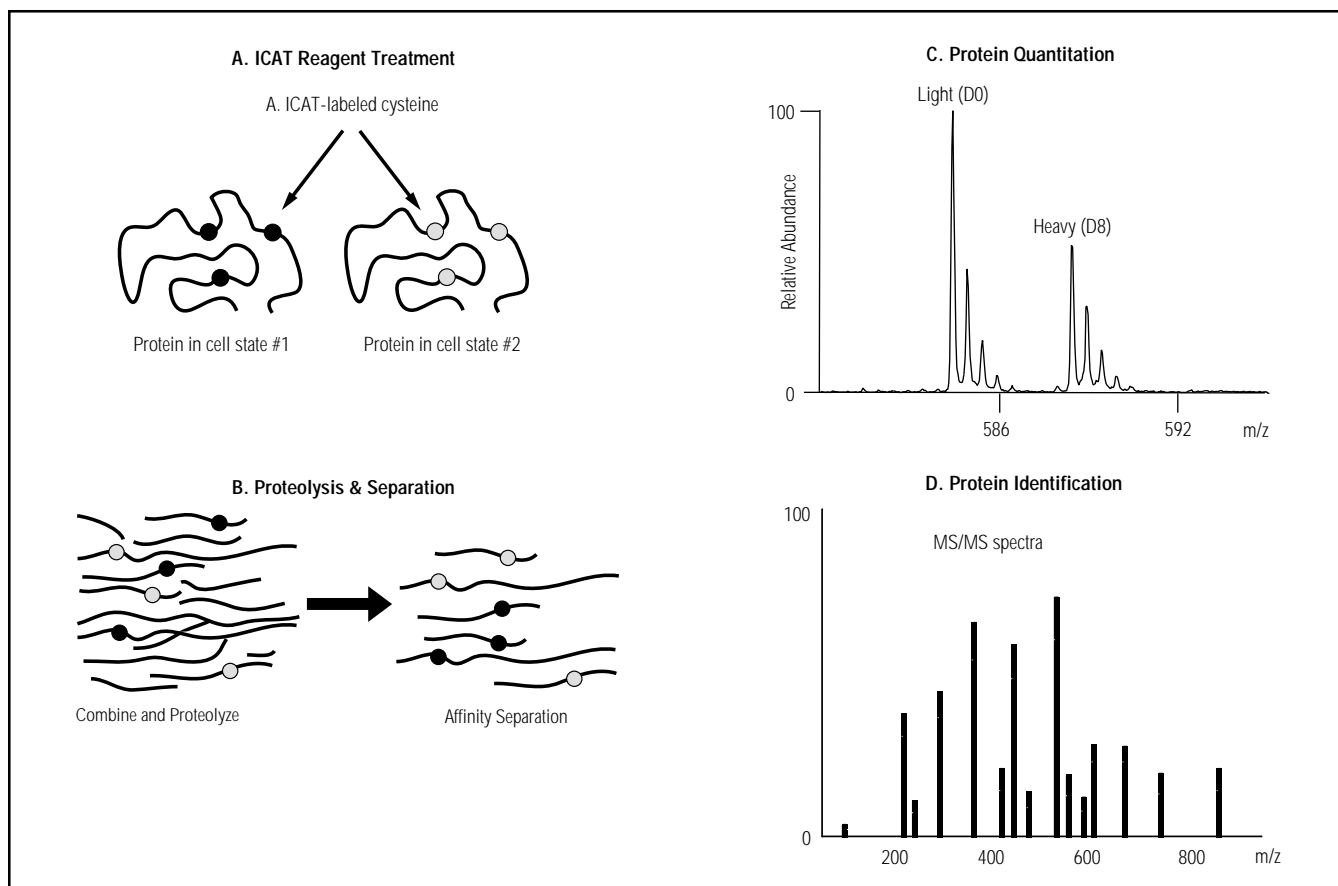


Figure 2. ICAT strategy.¹

In this application an UltiMate Nano LC system, a Switchos II advanced micro column switching unit, a FAMOS micro autosampler and a Mariner-ToF mass spectrometer were used for the analysis of a six ICAT-labeled proteins mixture (Figure 3).

After a complete sample preparation according to the ICAT procedure, an aliquot of 2 μL containing about 2 pmol of labeled proteins was injected onto a strong cation exchange micro-column (300 μm I.D. x 5 mm Spherisorb, 5 μm) at a flow rate of 20 $\mu\text{L}/\text{min}$. To release the peptides several plugs of increasing concen-

trations in salt are injected in series with the FAMOS autosampler. The saline solution was made of a 5 mM potassium phosphate buffer (pH=3) containing various amounts of potassium chloride (i.e., 0–250 mM). Each peptide fraction is then on-line concentrated and desalted on a reversed-phase precolumn before being backflushed on an analytical column. Separation is achieved on a nano LC column (75 μm I.D. x 150 mm) packed with Vydac C18 (5 μm particles) at a flow rate of 250 nL/min with a gradient from 0% to 95% of 0.1% AcOH in acetonitrile in 30 min.

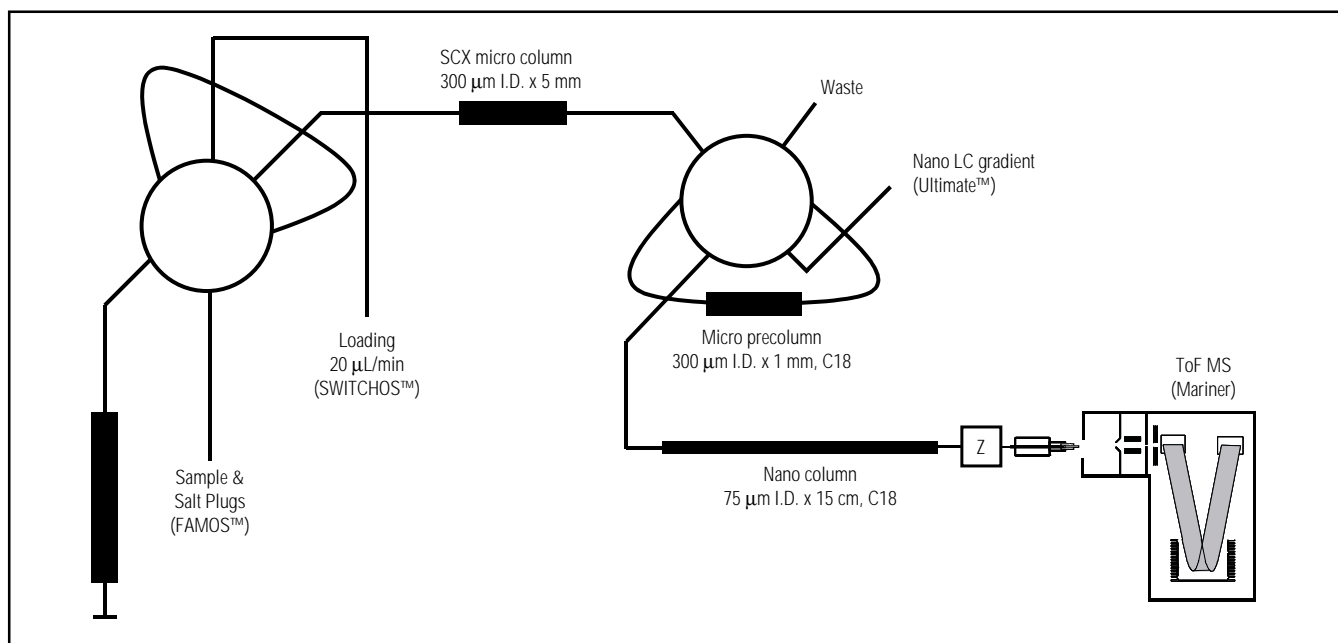


Figure 3. Experimental setup.

An example of the separation is shown in Figure 4 where spectrum A and B represents some examples of mass spectrometric data which could be used for proteins quantitation (Figure 5). With a Mariner ToF instrument, MS/MS spectra could not be taken which prevents identification of the proteins.

REFERENCES

1. S. Gygi, B. Rist. et al., *Nat. Biotechnol.* 17 (1999), 994-999.
2. T. Griffin, R. Aebersold, University of Washington, Seattle (USA)

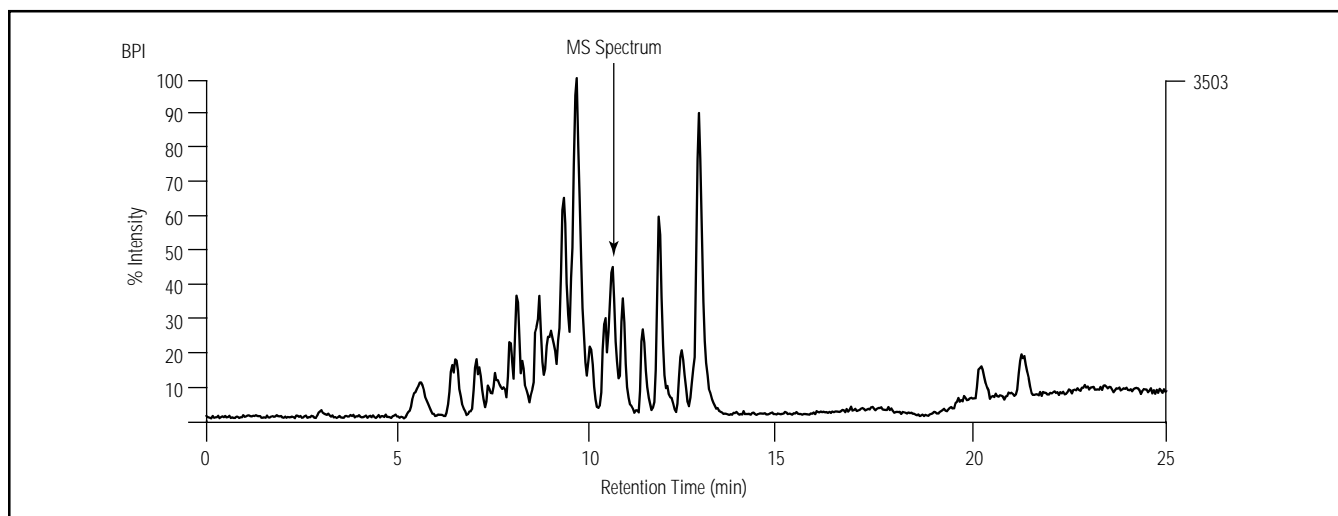


Figure 4. C18 LC chromatogram obtained for a specific salt concentration on SCX column.²

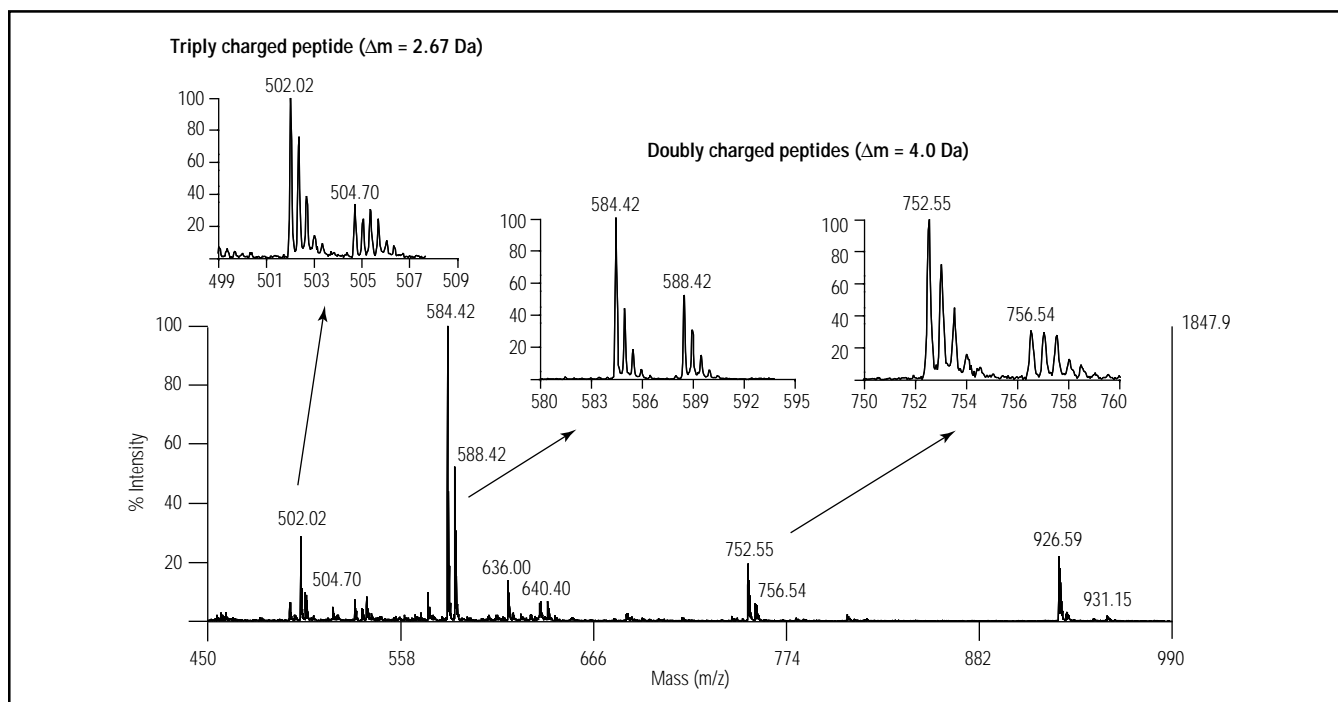


Figure 5. Example of MS spectra which could be used for proteins quantitation.²

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